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WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA 4, PA.

ACCOMMODATION IN THREE SINGLE MOTOR AXONS OF THE CRAYFISH CLAW

ERNEST B. WRIGHT AND WILLIAM J. ADELMAN
Department of Physiology and Vital Economics, The University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

THREE FIGURES

INTRODUCTION

In another paper it has been established that three motor axons, isolated in the crayfish claw and walking leg of the lobster, possess very different properties of excitation and conduction (Wright and Coleman, '54). The large fiber, fast closer, which elicits the twitch contraction of the adductor muscle of the claw has a higher absolute threshold than the small slow closer fiber connecting with this muscle. The fiber to the abductor muscle, opener fiber, is still more sensitive than the slow closer. The shapes of the respective strength-duration curves are different too, for with short duration stimuli, the fast closer fiber becomes the most sensitive of the three, which means that the excitability constants, k, differ from one fiber to the next. The refractory periods are consistently different also, and finally, when stimulated even the types of responses of the three fibers are not the same. The fast closer fiber conducts only one action potential during the passage of a prolonged D.C. stimulus, whereas the slow closer responds with a high frequency, short lasting, burst of spikes and the opener a long train of impulses lasting the duration of the stimulus and sometimes longer. Although the different threshold, k, and refractory period values of the respective fibers must play some part in creating the remarkably different types of responses of the three fibers, accommodation must also have a distinct

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role in the production of this behavior (Hill, '36). The present work was undertaken as a sequel to the previous one, to determine the value of the time constant of accommodation in each of the three motor fibers, fast closer, slow closer, and opener in the claw of the crayfish.

METHODS

The methods used were the same as before except for the addition of an RC circuit (Solandt, '36) to the Grass Stimulator. This simple circuit consisted of two 1 meg. resistors and a variable condensor, 0–1 microfarads. The resistors were connected in series to one side of the output of the stimulator, and the condensor across the output connecting the mid-point between the two resistors to the other output lead. The output square wave could be "blunted" by this circuit so that the initial time rise (T) of the square wave from 0 to the set voltage could be varied from 0 to 250 msec. The value E/E_o, where $E_o =$ absolute threshold for long duration shocks at $C = O$, and $E =$ threshold for shocks at different C values, was plotted against various T settings, where $T = RC$, and the time constant calculated as the reciprocal of the slope of this plot (see Hill, '36; and Solandt, '36).

The crayfish, Cambarus bartoni, was used in all but a very few experiments in which the giant southern crayfish, Cambarus clarkii, was used. The preparations and solutions were all as described before (Wright and Coleman, '54).

The recording and stimulating leads were also as described before except the stimulating leads were plated with platinum black to lower the impedance. This allowed for stimulation at lower voltages and markedly reduced the shock artifact from the long duration slowly rising pulses. The recording leads were connected directly to one side of the Dumont 322 twin beam oscilloscope. This machine contains a D.C. amplifier with cathode follower input, which at full gain will produce a 5-inch deflection for 10 mv. signal and a drift of about 1 mv. per hour. Since all stages use A.C.

TABLE 1

Accommodation, λ, and excitability, k, time constants and repetitive discharge response of single crayfish motor axons

FAST CLOSER			SLOW CLOSER			OPENER		
λ msecs.	$\frac{k \times 10^3}{\text{sec.}}$	Repetitive	λ msecs.	$\frac{k \times 10^3}{\text{sec.}}$	Repetitive	λ msecs.	$\frac{k \times 10^3}{\text{sec.}}$	Repetitive
9.0	. .	—	9.3	. .	—	7.4	. .	—
4.0	1.69	—	11.0	. .	+	45.0	0.85	++ [1]
0.8	. .	—	9.0	1.75	—	140.0	. .	+++
1.8	. .	—	4.0	. .	—	11.4	. .	—
2.0	3.08	—	21.0	. .	—	37.5	1.13	+
16.6	. .	—	25.0	1.58	+	85.0	0.48	+++
13.7	. .	—	7.3	1.71	—	26.0	1.39	— [2]
6.3	2.25	—	25.5	. .	+	33.3	0.74	+
7.5	3.18	—	23.3	. .	+			
17.5	2.20	+	22.5	1.43	+			
3.0	1.68	—	5.5	. .	—			
11.0	2.67	—						
7.76	2.39	—	14.85	1.62	+	48.2	0.92	++

[1] See table 4 and text.

[2] Abnormally high threshold for some reason prevented repetitive firing.

for tube heaters, a 1–2 mv. ripple is unavoidable in the baseline at the highest gain. The action potentials recorded were all 25 mv. or greater so that only moderate gain was necessary and the A.C. ripple was negligible.

RESULTS

The data presented in table 1 shows the remarkable differences between the time constants of accommodation of the three fibers. The fast closer lambda is 7.8 msec., the slow closer 14.9 msec. and the opener 48.2 msec., mean value. In some cases strength-duration curves were taken and k calculated and listed. The ability of a fiber to fire repetitively with long duration D.C. pulses is indicated qualitatively by plus signs. With two exceptions repetitive firing occurred always in fibers with a time constant greater than 22 msec. One exception is the slow closer showing repetitive responses with a lambda of 11 msec. The other exception is an opener fiber with a lambda of 26 msec. and no repetitive discharging. The latter was remarkable for an unusually high threshold and large k value which may have been caused by damage in preparation.

TABLE 2

Accommodation time constant, λ, values in slow closer fibers which fire repetitively or do not fire repetitively

REPETITIVE		NON-REPETITIVE	
Slow closer	Opener	Slow closer	Opener
ms.	*ms.*	*ms.*	*ms.*
11.0	45.0	9.3	7.4
25.0	140.0	9.0	11.4
25.5	37.5	4.0	26.0
22.5	85.0	21.0	
	33.3	7.3	
		5.5	
21.0	68.1	9.4	14.7

The time constants of accommodation of the slow closer and opener fibers showing repetitive responses are separated from those showing no repetitive firing in table 2. Evidently an accommodation time constant of 21 msec. or greater is required for the repetitive discharge.

Many double fiber preparations were made and the results were in good agreement with the means tabulated from the single fiber experiments. A typical fast-slow closer double preparation experiment is shown in figure 1. The upper curve, open circles, is the data from the fast closer and the

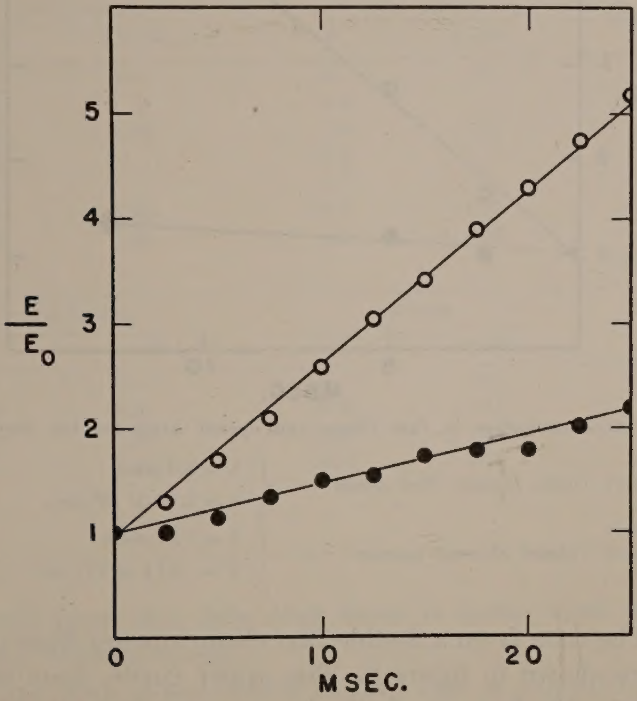

Fig. 1 Accomodation in fast closer and slow closer axons of the crayfish.

Upper curve (open circles) fast closer $\left\{ \begin{array}{l} \lambda = 6.3 \text{ msec.} \\ k = 2.25 \times 10^3/\text{sec.} \end{array} \right.$

Lower curve (closed circles) slow closer $\left\{ \begin{array}{l} \lambda = 22.5 \text{ msec.} \\ k = 1.43 \times 10^3/\text{sec.} \end{array} \right.$

lower curve from the smaller fiber. The difference in accommodation is clear, the actual time constant values are 6.3 msec. for the fast closer and 22.5 for the slow closer. The k values are 2.3 for the fast closer and 1.4 for the slow closer. The data from a double fast closer-opener fiber preparation are shown in figure 2. The upper curve, open circles, is data from the fast closer, the lower curve from the opener. The time constant values are even more separated in this paired experiment. The lambda for the closer is 3.0 msec. and 33.3 msec. for the opener fiber.

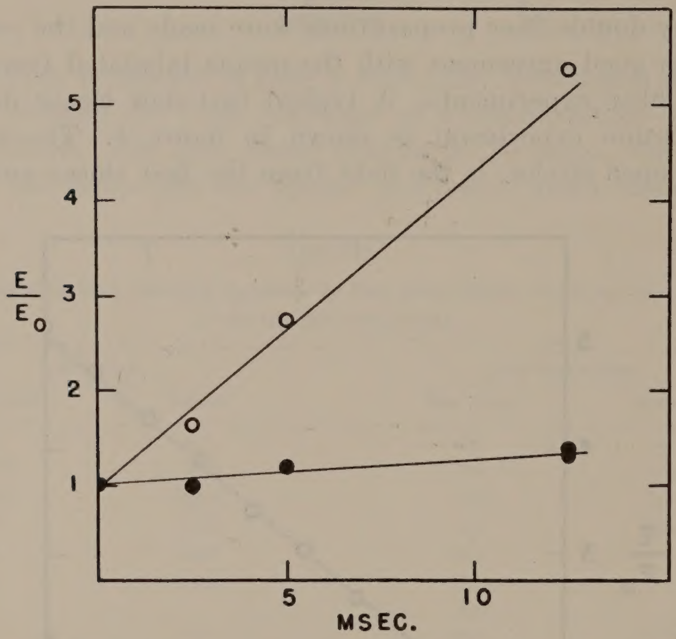

Fig. 2 Accommodation in fast closer and opener axons of the crayfish.

Upper curve (open circles) fast closer $\left\{ \begin{array}{l} \lambda = 3.0 \text{ msec.} \\ k = 1.68 \times 10^3/\text{sec.} \end{array} \right.$

Lower curve (closed circles) opener $\left\{ \begin{array}{l} \lambda = 33.3 \text{ msec.} \\ k = 0.74 \times 10^3/\text{sec.} \end{array} \right.$

The k values are 1.7 and 0.7 × 10^3/sec. respectively. The lambda data from all the paired observations are presented in table 3. The double preparations were either fast and slow closer fibers or fast closer and opener fibers, and the per cent differences of the time constants of accommodation are listed. Between the two closer motor fibers the per cent difference is 195% and between fast closer and opener fibers it is 458% mean values. Although no paired observations were made with slow closer and opener fibers, it can be inferred from this data that there is better than 200% difference between them.

TABLE 3

The accommodation time constant, λ, values from paired observations with fast and slow closer fibers and with fast closer and opener fibers in the crayfish

FAST CLOSER		SLOW CLOSER		% DIFFERENCE
λ	$k \times 10^3$	λ	$k \times 10^3$	
ms.	*sec.*	*ms.*	*sec.*	
0.8	...	4.0	...	400
2.0	3.08	7.3	1.71	265
16.6	...	25.5	...	54
13.7	...	23.3	...	70
6.3	2.25	22.5	1.43	257
4.0	1.69	9.0	1.75	125
				Average 195%

FAST CLOSER		OPENER		% DIFFERENCE
λ	$k \times 10^3$	λ	$k \times 10^3$	
ms.	*sec.*	*ms.*	*sec.*	
11.0	2.67	37.5	1.13	241
17.5	2.20	26.0	1.39	49
3.0	1.68	33.3	0.74	1085
				1375
				Average 458%

It has been a consistent finding that a repetitively firing fiber will become non-repetitive for a long time before it becomes altogether non-functional (Hodgkin, '48). A typical example of what happens to the lambda and k values in an excellent opener fiber preparation after 45 minutes, in this case, is shown in figure 3. The initial values for lambda and k, lower curve, open circles, are 85 msec. and 0.48×10^3/sec. respectively, and the fiber was highly repetitive in responding and had a low threshold of 24 mv. Forty-five minutes later the threshold was still low, 42 mv., but lambda, upper curve, solid circles, had decreased to 25 msec. and the

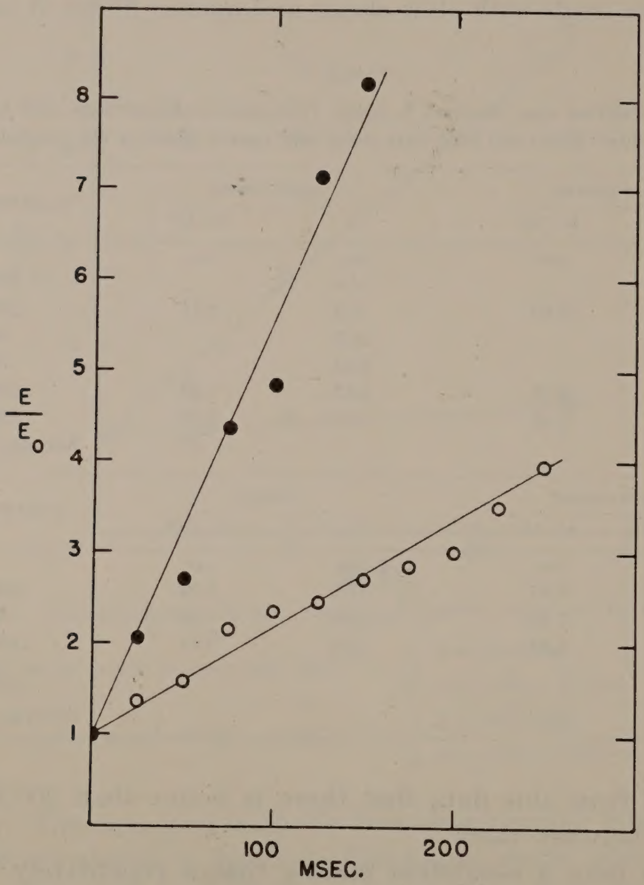

Fig. 3 The effect of time on accommodation in the opener fiber of the crayfish.

Lower curve (open circles) taken immediately after isolation of the fiber which responded with repetitive discharges.

Upper curve (closed circles) taken 45 minutes later when the fiber no longer responded repetitively.

k value was now 1.3 × 10^3/sec. The fiber response was, at this time, only slightly repetitive.

Another experiment with an excellent opener fiber preparation demonstrates that the lambda value does not always decrease with time (table 4). The fiber was at first repetitively responding and the lambda value was 45 msec., but after 10 minutes of almost continual electrical manipulation for obtaining the time constants, lambda was reduced in magnitude to 27 msec. and the fiber responded with only one spike, with long duration D.C. stimuli. The accommodation at this time was following the usual course. After a 10 minute "rest," the fiber response was again repetitive and the lambda value was 70 msec. This value dropped a little to 59 msec. in the next 10 minutes, but then after a 20 minute interval of no stimulation, three lambda values were taken and found to be 100, 110, and 250 msec. After two hours, the lambda value was 215 msec.; after 4 hours, 230 msec.; and the experiment was terminated after 6 hours with the fiber still normally functional.

TABLE 4

The effect of time on the time constant of accommodation, λ, value in the opener fiber of the crayfish claw

TIME	λ MS.	REPETITIVE RESPONSE
11:0.5 A.M.	45	++
11:15	27	—
11:25	70	+++
11:35	59	+++
11:55	100	++++
	110	
	250	
1:10 P.M.	215	++++
3:00	230	++++

Fiber still responding the same when experiment terminated at 5:00 P.M.

DISCUSSION

The accommodation time constants of the three crustacean motor fibers are very different. This was not an unexpected finding and indeed other results than these would have been difficult to fit with the previous results on repetitive firing and excitability (Wright and Coleman, '54). The fast closer fiber accommodates very rapidly, the slow closer rather slowly and the opener fiber hardly at all. Whether or not accommodation is a real physiological property of nerve *in vivo* is a question often argued. The consistent results reported here show definitely that under identical conditions these three motor fibers possess quite different abilities to become insensitive to a continuous stimulation. If this is so *in vitro* with applied electric current stimulus, why should it also not be true *in vivo* with bioelectric current stimulation? The fact is that the stimulation voltage used here was usually of the same order of magnitude as the action potential, sometimes even less, and assuming similar membrane electrical constants for fibers *in vivo* or *vitro,* the stimulating currents are about the same as generated by the action potential explosion itself. It seems, therefore, that if the electrical theory of conduction in a fiber is valid, that these three crustacean motor fibers should accommodate to bioelectrical excitation *in situ* in the same way as they, after isolation, accommodate to applied electrical pulses. The three fibers can be likened to three sensory endings in mammals in their respective accommodation differences. The touch endings accommodate rapidly, temperature sensitive endings accommodate more slowly, and pressure endings accommodate very slowly indeed as do muscle proprioceptors.

Now, the question is, what is the cause of the difference in accommodation in these three fibers. Of course, size may have some bearing on this problem in that the most rapidly accommodating fiber is also the largest of the three. However, the other two smaller fibers are nearly the same size but their respective lambdas are very different, and furthermore, the fiber which is usually the larger of the two possesses the lower accommodation value in this case.

It is interesting to note that the lambda values decrease as the fiber loses the repetitive firing property with time. It can be said here, too, that the fibers with the smallest lambdas originally, the fast closers especially, are the most difficult to maintain *in vitro* for long periods of time. Thus it might be surmised that the fast closer is normally maintained in a state through which the slow closer and opener fibers pass as they deteriorate with time. Much continuous stimulation also produces this reduction of lambda value and cessation of repetitive firing. This means that fatigue is marked by an increase in accommodation. Continuous stimulation results in a leakage of potassium from the inside of the crustacean axon (Young, '38; Hodgkin and Huxley, '46; Keynes, '50; Cowan, '34; Arnett and Wilde, '41). In the single fiber preparation which is raised up in oil and therefore is surrounded by only a microscopic film of solution, a small amount of potassium leaking out of the fiber during activity could lower the concentration gradient across the membrane immersed in oil rather easily and, perhaps markedly, after long periods of excitation. A simple calculation can be made using the recent data of Hodgkin and Huxley ('46), and also Keynes ('50). The volume of external saline solution surrounding 1 cm length of single axon raised in oil is about 3×10^{-6} cm^3. The potassium concentration is about 5.0 mM in the crayfish solution so that the total amount of external potassium is about 1.5×10^{-11}. If 2×10^{-14} moles per impulse per centimeter length of potassium are released during excitation, about 1500 impulses are necessary to double the external potassium concentration. Such an increase in the external potassium ion concentration has been found to reduce the lambda value of frog sciatic 60 to 80% (Solandt, '36). Just a few volleys of impulses or several seconds' continuous stimulation can bring a lambda value of a repetitively firing fiber down below the 22 msec. value required for the multiple discharging. A 600% potassium concentration increase can produce a twofold rise in the k constant value of frog sartorius muscle in just a few minutes (Carleton, Blair and Latchford, '38). It is reasonable to suppose that doubling the concentration of the ion around a single nonmyelinated axon can have the same effect even more quickly. After long times an isolated nerve loses small amounts of potassium to the surrounding medium (Fenn and Gerschman, '52; Keynes, loc. cit.) even without stimulation, and as already pointed out, as time passes, the lambda value is reduced and accommodation increases. It should be pointed out also that in the few preparations in which k values were calculated to check with the k values previously reported (Wright and Coleman, loc. cit.), that the k value is increased with time and fatigue, so that the opener fiber after a long time, for example, becomes identical in lambda and k values to the normal fast closer. The problem of the effect of ion concentration in the external medium, potassium especially, on the k and lambda values of these three fibers is now being investigated.

In the present work, only the crayfish fibers have been studied for accommodation differences, whereas in the previous experiments (Wright and Coleman, '54), crayfish and lobster nerve fibers were used. It was observed that the lobster fast closer fiber sometimes fired repetitively, but the crayfish fast closer fiber never did. It is interesting to bear in mind that the lobster nerve fibers were prepared in the walking leg whereas the crayfish fibers were prepared in the claw. The pincers of the walking leg are not defense weapons or nutriment grabbers, but are used for clinging and hence do not require as fast a "snap shut" reaction. In fact a slow maintained contraction should be more useful with this limb. Another problem being looked into at this time is the difference, if any, between fast closer fibers in the claw and in the walking leg of the lobster.

The results of the whole series of investigations may be summed up as follows. The three motor fibers of the crustacean differ in their excitation properties. The fast closer which causes a twitch contraction of the adductor muscle of the pincer has a small lambda value, a large k value, a long refractory period and a high threshold. This fiber will therefore adapt quickly and not tend to fire repetitively. The slow closer which elicits a strong contracture of this muscle possesses a lambda of larger value bordering on 22 msec. necessary for repetitive firing, a smaller k, a very short refractory period and a low threshold. This fiber will fire repetitively because it adapts slowly, and due to the short refractory period and moderately large k and low threshold, will fire at high frequencies. The opener which produces a contracture of the abductor muscle of the pincer, hardly adapts at all, has a small k, a short refractory period and a very low threshold, and it will therefore fire repetitively for long times at moderate frequencies.

These differences in accommodation may be of extreme value to the animal, for a very small stimulus could effectively open the claw preparing the pincers to "grab" an object. More rapid adaptation to closing stimuli is certainly necessary for release by the pincers because the closing muscle is so much stronger than the opening muscle. It should be mentioned here that peripheral inhibition (Wiersma, '41) also must play an important part in these coordinated movements, but has not been included in the present investigation.

SUMMARY

It has been found that the accommodation time constant values of the three motor fibers fast closer, slow closer and opener of the crayfish claw are different.

The fast closer has a lambda value of 7.8 msec., the slow closer of 14.9 msec., and the opener of 48.2 msec., on the average. Paired observations substantiated these values.

In paired observations the average slow closer lambda was 195% larger than the average fast closer lambda; the average opener lambda was 458% larger than the average fast closer lambda.

The lambda value decreased with time and with continuous stimulation or fatigue.

Some reasons for these differences are discussed.

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EXCITATION AND CONDUCTION IN CRUSTACEAN SINGLE MOTOR AXONS

ERNEST B. WRIGHT AND PAUL D. COLEMAN
Department of Physiology of the University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

FOURTEEN FIGURES

INTRODUCTION

When the claw or limb is removed from crayfish or lobster by cutting through the ischyopodite joint nearest the main body of the animal, a sequence of three movements by the pincers of the excised member always occurs. The pincers of the excised limb, normally held partly open in the relaxed position, first snap firmly closed and remain closed for a brief time, then open wide and maintain this position for a long time, perhaps several minutes, and finally, assume the partly open relaxed position again. These movements are of course caused by the indirect mechanical stimulation of the nerve to the pincer muscles when it is cut through at the ischyopodite joint. This stimulus appears to elicit first a contraction of the large adductor muscle, then contraction of the small abductor muscle in the propodite section, and then finally both muscles relax. What is more likely, however, is that a simultaneous contraction of both muscles takes place immediately upon cutting the nerve. The weaker abductor muscle contraction is the more sustained and therefore becomes apparent only after the stronger and shorter lasting adductor muscle contraction is terminated.

Only three motor fibers in the whole nerve are involved in this series of movements (Wiersma, '41). The "fast closer" fiber with a single impulse elicits the twitch contraction of the adductor muscle, the "slow closer" fiber which with volleys of impulses produces a strong sustained "contracture" type shortening of this muscle, and the "opener" fiber which with volleys of impulses causes a slow contracture type reaction of the abductor muscle are the three fibers concerned. Histologically these fibers have been shown to anastomose over their respective muscles forming single motor units (van Harreveld, '37; Wright and Wiersma, '46). Now all three fibers receive the identical mechanical stimulus in the separation of the limb from the animal. Therefore to account for the differences in the contraction times of the two single motor units, adductor and abductor muscles, the fast and slow closer nerve fibers must respond with a brief volley of impulses at most and the opener fiber with a much longer volley of action potentials. Wiersma and van Harreveld ('36) have already shown that the slow closer fiber can respond with up to 32 impulses at 200/sec. during weak D.C. stimulation, and that the fast closer is much less apt to fire repetitively. It has also been shown that the three fibers differ in thickness, the fast closer fiber being 58 μ, the opener fiber, 41 μ and the slow closer fiber, 36 μ in diameter on the average (Wiersma and van Harreveld, '38). Thus the smaller fibers tend to discharge repetitively more readily than the large fiber, but there must also be a large difference between the type of repetitive firing of the two smaller fibers. The larger of these two fibers, the opener, must respond with very prolonged repetitive firing compared to the smaller closer fiber.

Arvanitaki ('38) using whole crustacean nerve, and more recently Hodgkin ('48) with Carcinus single axons, have studied repetitive firing and the latter has classified unmyelinated crustacean fibers into three groups. The first group includes those axons which when stimulated with a D.C. pulse respond with short duration high frequency bursts of impulses. The second group contains the axons which fire repetitively at low frequencies but for relatively long times, and the third group includes axons which have a higher threshold and rarely respond with repetitive discharges. Perhaps the three crustacean motor fibers involved in the opening and closing of the pincers may belong each to one of these groups.

The present investigation was undertaken to find out if these three motor fibers connecting with the muscles in the propodite section of the limb do possess different properties of excitation and conduction to explain the different responses of the fibers to the same stimulus.

METHODS

The crayfish Cambarus bartoni which is found in the fresh water streams around Rochester and the lobster Homarus Americanus purchased at the fish market were used. The lobsters were kept in aerated sea water while the crayfish were maintained in aerated fresh water both at 5°C.

The claw of the crayfish or the walking limb of the lobster was separated from the animal by cutting through the ischyopodite segment, and the nerve in the meropodite section exposed (Wiersma and van Harreveld, '36). Five to 20 mm of single motor axon was isolated and immersed in either sea water for the lobster preparation or van Harreveld ('36) solution for crayfish preparations, at 20–22°C. When the axon was isolated, the stimulating and recording electrodes were manipulated well under the axon and most of the solution pipetted off until the axon just rested on the electrodes at the surface of the fluid. Liquid petrolatum was then floated over the surface of the solution and the axon was carefully raised with the electrodes into the oil forming a sharp meniscus along the length of the axon at the oil-water interface. The meniscus was broken by gentle probing with a needle beneath the axon and the fiber was then free in oil on the electrodes although it was still connected at both ends to muscle and connective tissue of the meropodite section. In many cases two or all three axons were prepared in one limb. When in oil, the fiber was stimulated and the action potential and muscle contraction observed simultaneously in order to assure the fiber to be in perfect working order and to identify the action potential with the proper fiber by its function. The fiber was then cut at either end so as to be completely isolated on the electrodes. This eliminated practically all artifacts from stimulus current escape or extraneous pickup.

When the fiber had been identified and isolated, the procedure was usually as follows. First the fiber threshold was tested with different strengths and durations of current. With the long duration shocks the repetitive firing, if present, was recorded. Then the refractory period was studied using two short duration shocks at varying time intervals, and at the same time the change in latency and magnitude of one action potential initiated during the relative refractory state was recorded. This type of experiment was carried out sometimes on two or even all three fibers at once.

The electrodes were made of fine platinum wire which was fastened in holders connecting with micromanipulators. The wire was flamed before each experiment to eliminate contamination by oil, foreign material and oxide coating. The stimulating leads were each separated by about 2–3 mm as were the recording leads, and the distance between recording and stimulating electrodes was variable from 0–7 mm.

Stimulation was produced by D.C. pulses emanating from either a Grass Model 3C stimulator or from a previously described electronic circuit (Wright, '49). Another electronic device (Wright, '49) was sometimes included in the circuit to break up the square wave into two short duration pulses with variable time interval between them for the refractory period studies.

Recording was obtained through an Offner 140 capacity coupled preamplifier and a Dumont 304H oscilloscope.

RESULTS

The results of all the data from crayfish and lobster nerve fibers are presented in tables 1 to 4. These results will be described below according to the organization of the tables.

Threshold. In table 1 the mean threshold voltage of the fast closer crayfish fibers, 81 mv., is slightly larger than that of the slow closer, 70 mv., and considerably greater than that of the opener, 41 mv. On the basis of the most sensitive fibers

TABLE 1

Excitation and conduction properties of crayfish single motor axons

FIBER TYPE	THRESHOLD MILLIVOLTS		k × 10^3/sec.	REFRACTORY PERIOD MILLISECONDS		REPETITIVE DISCHARGE RATE PER SEC.		% SIZE DECREASE		% LATENCY INCREASE	
	Mean	Range		Mean	Range	Mean	Range	Mean	Range	Mean	Range
Fast closer	81	58–115	3.94	2.10	1.6–2.6	0	0	13.2	0–30	50.6	14–100
Slow closer	70	42–107	2.82	1.64	1.2–2.4	196	100–325	16.7	0–33	54.5	29–133
Opener	41	20– 76	1.13	2.24	1.2–3.0	255	200–325	28.4	4–64	52	13–167

TABLE 2

Excitation and conduction properties of lobster single motor axons

FIBER TYPE	THRESHOLD MILLIVOLTS		k × 10^3/sec.	REFRACTORY PERIOD MILLISECONDS		REPETITIVE DISCHARGE RATE PER SEC.		% SIZE DECREASE		% LATENCY INCREASE	
	Mean	Range		Mean	Range	Mean	Range	Mean	Range	Mean	Range
Fast closer	64	50– 75	2.30	2.46	1.6–4.4	208	65–235	18.4	0–43	52	15–100
Slow closer	58	28–115	1.59	2.10	1.2–3.2	225	65–350	23.8	0–90	80	7–650
Opener	67	6–150	1.50	2.54	1.4–3.6	170	100–450	17.6	0–43	70.6	0–140

TABLE 3

Excitation and conduction properties of crayfish single motor axons double and triple fiber preparations

EXPERIMENT	THRESHOLD MILLIVOLTS			k × 10^3/sec.			REFRACTORY PERIOD MILLISECONDS			REPETITIVE DISCHARGE RATE PER SEC.			% SIZE DECREASE			% LATENCY INCREASE		
	Fast	Slow	Opener	F	S	O	F	S	O	F	S	O	F	S	O	F	S	O
1	115	107	..	..	..	..	..	..	..	None	..	..	..	..	..	..	..	..
2	5500	2700	..	3.1	1.6	..	..	..	..	None	..	..	..	..	..	..	..	..
3	180	90	..	1.3	1.6	..	..	..	..	None	..	..	..	..	..	..	..	..
4	710	560	..	2.4	1.5	..	..	..	..	None	..	..	..	..	..	..	..	..
5	140	..	65	2.6	..	1.1	..	..	..	None	..	..	..	..	..	..	..	..
6	150	..	75	2.4	..	0.63	..	..	..	None	..	..	..	..	..	..	..	..
7	75	..	85	3.3	..	1.8	..	..	..	None	..	..	..	..	..	..	..	..
8	..	..	..	6.2	4.5	..		..	..	None	..	..	..	..	..	..	..	..
9	..	..	..	6.7	4.6	..	..	..	..	None	..	..	..	..	..	..	..	..
10	82	60	..	4.1	2.4	..	..	..	..	None	..	..	..	..	..	..	..	..
11	108	95	..	2.7	1.7	..	2.6	1.8	..	None	175	..	13	20	..	116	66	..
12	..	..	..	..	..	..	2.2	1.2	..	None	325	..	30	..	..	60	..	..
13	..	..	..	..	..	..	1.6	1.4	..	None	200	..	27	..	..	..	..	..
14	..	..	..	..	..	..	1.6	1.4	..	None	..	..	6	..	..	133	100	..
15	..	..	..		..	..	2.4	1.4	..	None	100	..	..	0	..	71	71	..
16	..	..	..	..	..	..	1.8	1.4	..	None	..	..	15	..	..	28	14	..
17	..	..	..	..	..	..	2.0	2.0	..	None	175	..	19	..	..	100	50	..
18	..	..	..	..	..	..	1.8	1.4	..	None	250	..	..	33	..	50	..	..
19	..	..	..	..	..	..	2.4	2.0	2.8	None	..	325	10	18	15	..	70	13
20	..	..	..	..	..	..	2.6	1.6	..	None	..	..	0	28	..	100	..	..

TABLE 4

Excitation and conduction properties of lobster single motor axons double and triple fiber preparations

EXPERIMENT	THRESHOLD MILLIVOLTS			k × 10³/sec.			REFRACTORY PERIOD MILLISECONDS			REPETITIVE DISCHARGE RATE PER SEC.			% SIZE DECREASE			% LATENCY INCREASE		
	Fast	Slow	Opener	F	S	O	F	S	O	F	S	O	F	S	O	F	S	O
1	75	45	..	1.7	1.0	..	..	..	..	..	..	..	..	..	..	..	..	..
2	60	70	..	4.2	4.5	..	..	..	..	..	..	..	..	..	..	..	..	..
3	63	58	..	3.4	2.1	..	..	..	..	..	..	..	..	..	..	..	..	..
4	50	35	..	2.1	1.3	..	..	..	..	..	..	..	..	..	..	..	..	..
5	63	55	..	2.7	3.0	..	..	..	..	..	..	..	..	..	..	..	..	..
6	115	..	110	0.9	..	1.7	..	..	..	..	..	..	..	..	..	..	..	..
7	400	..	350	3.2	..	3.1	..	..	..	..	..	..	..	..	..	..	..	..
8	..	..	..	..	..	..	1.6	2.8	..	200	70	..	12	90	..	33	7	..
9	..	..	..	..	..	..	2.0	1.6	..	..	..	..	21	13	..	..	..	..
9	..	..	..	..	..	..	1.6	1.8	..	..	..	..	..	..	..	..	..	..
9	..	..	..	..	..	..	3.2	2.4	..	..	..	..	..	..	..	..	..	..
10	..	..	..	..	..	..	3.2	2.0	..	275	250	..	21	25	..	60	40	..
11	..	..	..	..	..	..	1.8	1.6	1.4	..	340	325	..	..	..	50	150	25
11	..	..	..	..	..	..	2.0	1.2	1.9	..	..	..	20	0	29	..	..	..
12	..	..	..	..	..	..	2.4	2.0	..	200	250	..	9	50	..	50	..	..
13	..	..	..	..	..	..	4.4	1.6	..	..	..	..	..	..	..	..	..	..
14	..	..	..	..	..	..	2.4	1.4	..	200	165	..	4	18	..	17	100	..
15	..	..	..	..	..	..	3.0	1.6	..	..	..	..	..	..	..	..	..	..
16	..	..	..	..	..	..	..	2.2	2.2	..	..	..	..	40	43	..	150	140
17	..	..	..	..	..	..	2.4	1.6	2.0	..	..	..	..	..	..	..	..	..
18	..	..	..	..	..	..	2.2	1.4	..	65	100	..	..	..	..	..	..	..
19	..	..	..	..	..	..	2.4	2.2	..	250	175	..	..	..	..	..	..	..

(which were usually the better preparations), this relationship is even more obvious. As can be seen in the range column (table 1) an opener fiber responded to as little as 20 mv. whereas the most sensitive slow closer required approximately twice this voltage, and the most sensitive fast closer required about three times as much voltage as the opener.

There are no such marked differences between the mean threshold values of the three motor fibers in the lobster (table 2). Again, however, on the basis of the most sensitive fibers, the threshold voltages conform to the same relationship described for the crayfish fibers. The most sensitive opener fiber required the least voltage, 6 mv. for stimulation. The most sensitive slow closer fiber threshold is more than 4 times as great, and the most sensitive fast closer fiber threshold is about 8 times as large.

The results of double and triple fiber preparations (tables 3 and 4) more clearly establish the threshold voltage relationship among the three types of fibers. In 9 crayfish preparations combining the fast closer with either the slow closer or the opener fibers, the threshold value of the fast closer was larger in every case except one. The same was true for 6 out of 8 similar preparations in the lobster.

The mean strength-duration curves for both lobster and crayfish fibers are shown in figure 1 (bottom). The points on the curves are calculated from 27 sets of data from slow closer fibers, 15 sets from opener fibers, and 13 sets from fast closer fibers. Typical strength-duration curves from a double fiber preparation (fast and slow closer fibers of the crayfish) are included in the upper part of the figure. The threshold value difference between fast closer fiber and the others is obvious in both graphs, but no distinction between the threshold voltages of the slow closer and opener fibers can be made. Another fact of interest shown very clearly in the figure is the difference in the shapes of the curves. Usually the curves cross, that is, the opener and slow closer fibers with the lower threshold voltages for infinitely long duration stimuli require more voltage to stimulate with short duration shocks than the fast closer

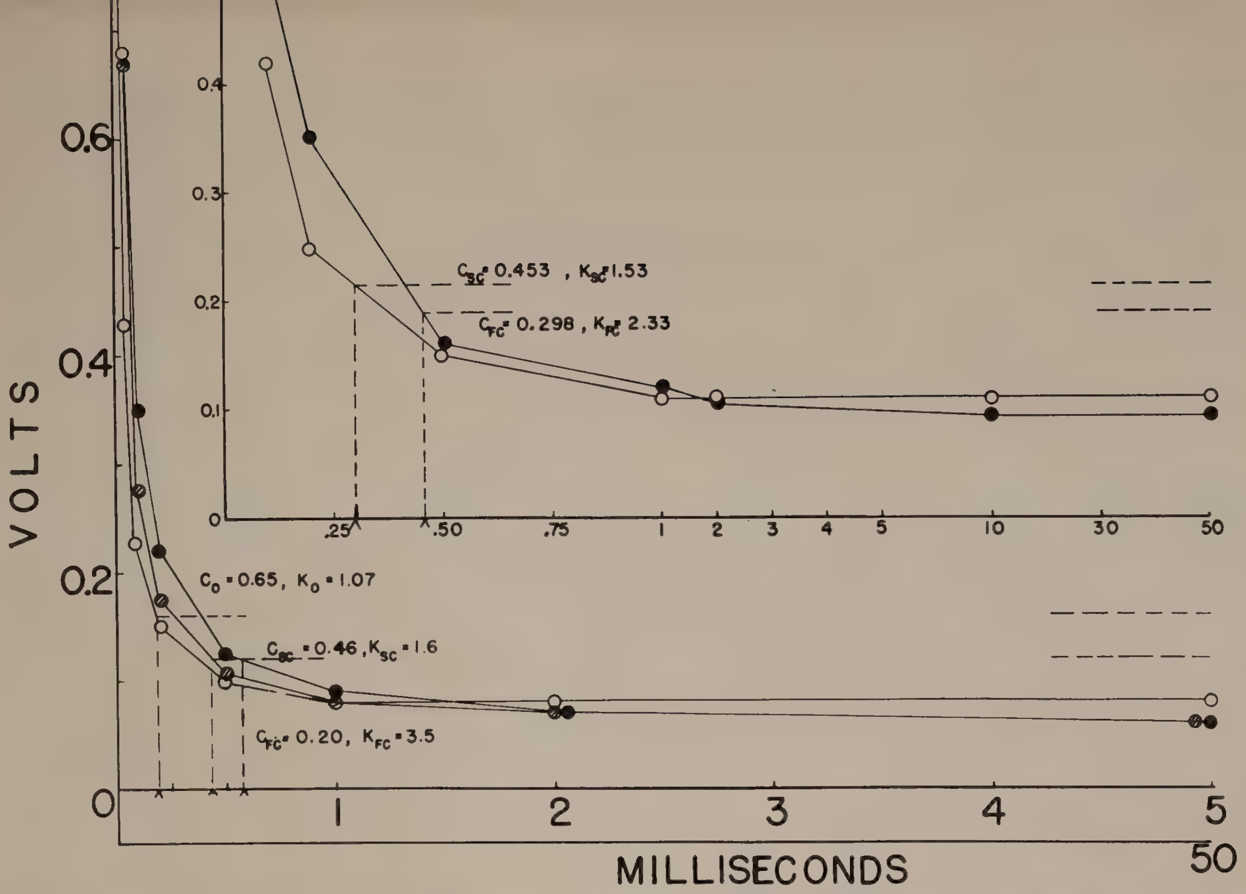

Fig. 1 Strength-Duration Curves of the three types of crustacean limb motor nerve fibers. The lower curves are drawn through the mean values of all results, the upper curves are results from a crayfish fast and slow closer double fiber preparation. Open circles indicate fast fiber closer, solid circles, slow closer; and shaded circles, opener fiber data. The chronaxes (C) and k (K) values for each fiber are shown with the sub letters FC = fast closer, SC = slow closer and O = opener. Abscissa = time in milliseconds, ordinate = stimulus voltage. The dashed lines show twice rheobase values used for determining chronaxes.

fiber. Thus the chronaxie of the small fibers is longer, and hence the excitability rate constant k shorter (Blair, '41) than for the fast closer fiber. The mean chronaxie values for all preparations are 0.20 ms. for fast closer fibers, 0.46 ms. for slow closer fibers, and 0.65 ms. for the opener fibers. The k figures are then 3.5, 1.6, and 1.07×10^3/sec. for the fast and slow closer, and opener fibers, respectively. The chronaxie values found in the typical fast and slow closer, double fiber preparations are 0.298 ms. for the fast, and 0.453 ms. for the slow fiber, and the k for the larger fast fiber is 2.33×10^3/sec. and for the slow, 1.53×10^3/sec.

k constant values were calculated by the equation $kt = \log_e \frac{v}{v-r}$ (Blair, loc. cit.) where t = time in seconds of stimulus duration, v = voltage applied, and r = rheobase voltage. The mean values are shown in column 4 of tables 1 and 2 for crayfish and lobster, respectively. It will be noted that for both species the k value for the large fast closer fiber is approximately one and one-half times the value for the slow closer fiber. The k value for the slow closer fiber of the crayfish is more than two times that for the opener fiber, but in the lobster these two k values are approximately the same.

In 9 out of 10 crayfish double fiber preparations this same k relationship is clearly shown (table 3), but in only 4 out of 7 lobster double fiber preparations does this relationship hold. It should be noted, however, that in the three lobster double fiber preparations which failed to produce the usual k relationship, the threshold ratios also deviated from the expected values.

Refractory period. The refractory periods of the different fibers also bear a relationship to one another. The smallest fiber, the slow closer, has the shortest refractory period, 1.64 ms. for crayfish and 2.1 ms. for lobster (mean values). Absolute minimum refractory period is 1.2 ms. for this fiber, and refractory periods as long as 2.4 ms. in crayfish and 3.2 ms. in lobster were recorded.

The largest fiber, the fast closer, has a mean refractory period of 2.1 ms. in crayfish and 2.4 ms. in lobster. The absolute minimum refractory period for this fiber is 1.6 ms. in both species, and refractory periods as long as 2.6 ms. in crayfish and 3.6 ms. in lobster have been measured.

The middle sized opener fiber has the longest refractory period, 2.24 ms. mean value for crayfish and 2.54 ms. mean value for lobster. Absolute refractory period values as low as 1.2 ms. for crayfish and 1.6 ms. for lobster were obtained from this fiber and refractory periods as long as 3.0 ms. for crayfish and 3.6 ms. for lobster have been recorded.

The refractory period results should really be divided into two groups, the refractory period of the local or initial response and the refractory period of the spike. This is illustrated in figure 2. The local response of a fast closer fiber of the crayfish makes its first appearance when the interval between two strong shocks has lengthened to about 1.2 ms., and then as the interval is further increased, the local potential increases in magnitude until finally the spike threshold is reached and the big action potential is triggered. This occurs when the interval between shocks is about 2 ms. The local potential is then approximately 15 mvs. in size, about one-third the size of the propagated response.

A similar recording from the opener fiber of the lobster is illustrated in figure 3. In this case, the recording lead is much closer to the stimulating one as indicated by the very small time delay between shock artifact and action potential. The local response can easily be detected as a hump in the lower half of the rising phase of both spikes in the upper picture. The stimuli strengths are just above threshold. When the interval between shocks is 2.5 ms., only the local response appears, but at 2.6 ms. the spike is just fired, but only after considerable latency (center figure). A 2 ms. interval causes the local response from the second shock to be markedly reduced in size below the spike threshold.

The effect of stimulus strength on the refractory period interval is well demonstrated in figure 4. Here the first column

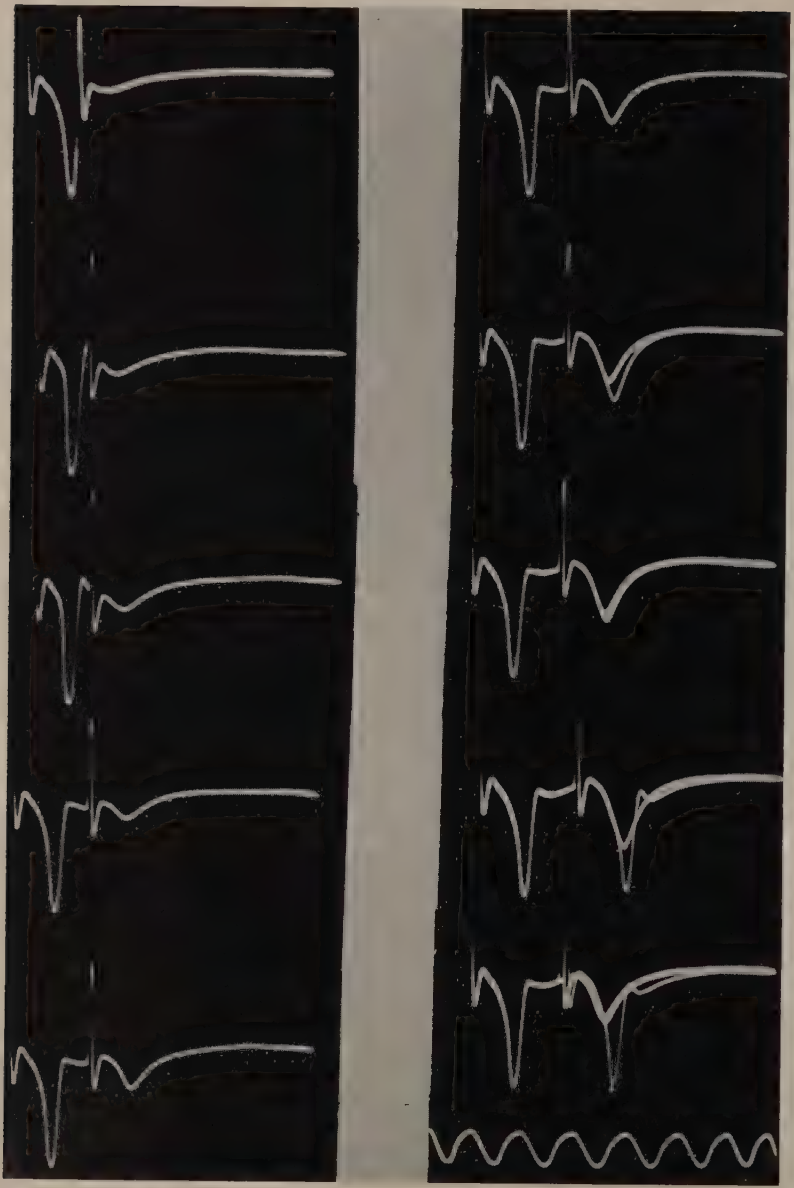

Fig. 2 Refractory period study on a crayfish fast closer fiber showing the effect of varying the time interval between two shocks. The initial diphasic deflection is the shock artifact followed by the action potential deflection downward, then the second shock artifact and the action potential elicited by it. 15 mv. 1000 ~ signal.

of action potential pictures, from a triple fiber preparation in the lobster, shows the time interval with the second shock at 10 times threshold strength. The opener fiber ceases to respond to the second shock when the interval is 2 ms., third photo down, the fast closer when the interval is 1.8 ms., and the small

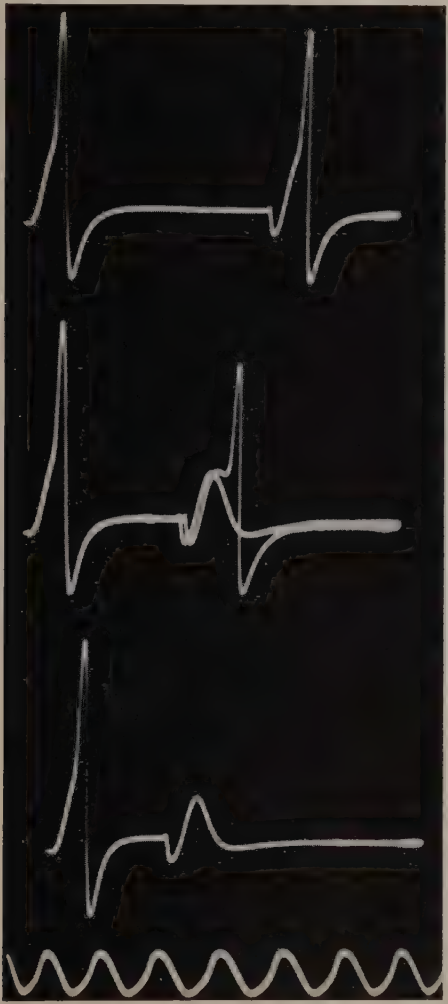

Fig. 3 Refractory period study of lobster opener fiber. Note the latency of the spike is greatly increased in the refractory state, center record. 1000 ~, 10 mv. timer.

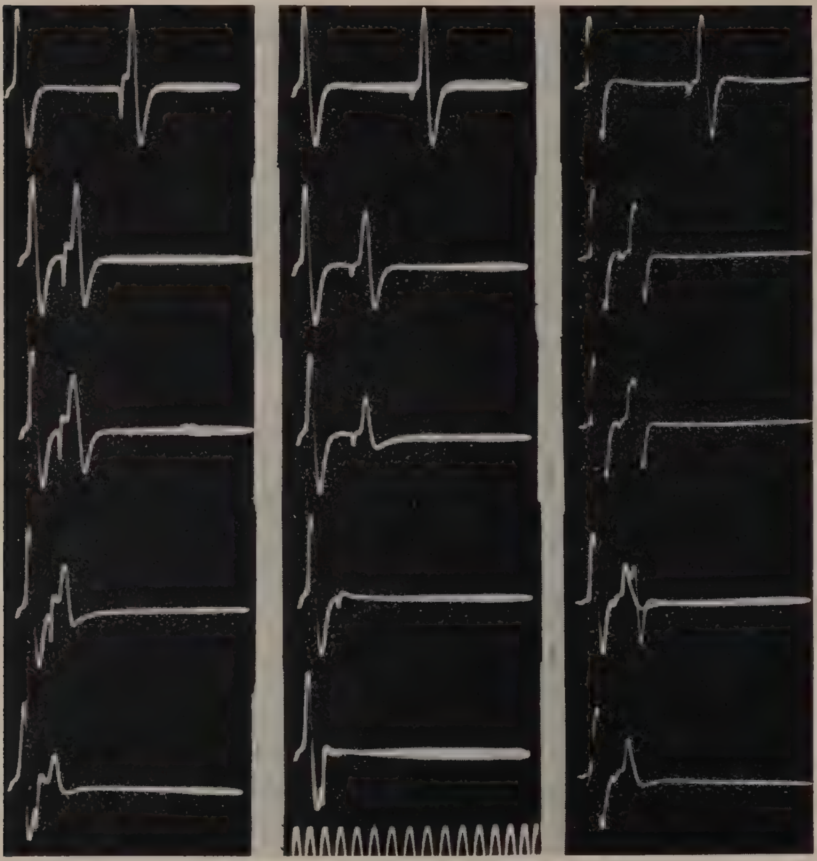

Fig. 4 Refractory period study on a lobster triple fiber preparation. Left column, with strong stimuli there is a decrease in the amplitude of the action potential elicited by the second shock as the interval between shocks is shortened. The initial decrease (second figure down) is small and is due to the decrease in the action potentials of the fibers in the relative refractory state, but there are sudden large decreases in the spike amplitude, third and fourth pictures down, as the opener fiber spike and then the fast closer fiber spike drop out when the second stimulus falls within the absolute refractory period of these fibers. Also there is a marked decrease in the size of the slow closer spike in the refractory state, bottom picture. Final recording of this series is at bottom of center column. Center column contains records of the same preparation using a weak stimulus. Right column shows records of refractory study on a lobster fast and slow closer preparation showing increase in latency of fast closer action potential. 1000 ~ signal, 20 mv. deflection amplitude as shown.

slow closer at the interval 1.5 ms., bottom picture of center column. With just threshold strength in the second shock, upper four pictures of center column, the interval between shocks at which the opener ceases firing a second time is 3.3 ms. The fast closer fiber no longer responds to the second stimulus after a 3.0 ms. interval, and the slow closer failed to conduct when the interval was 2.2 ms. These last figures probably represent the time interval of the relative refractory state for the three fibers, while the other figures using the strong second shock approximate the absolute period in these fibers. In the third column of figures is shown what happens to a lobster fast and slow closer double fiber preparation with decreasing time intervals between shocks. Both fibers fire in unison with large time intervals, top picture, but beginning at the 3 ms. time interval the action potentials begin separating because the latency for the fast closer action potential spike begins increasing. As the shock interval is made still smaller this latency continues to increase until at about 2 ms. shock separation, the fast closer fiber spike does not respond to every second shock. The slow fiber impulse dropped out when the interval became less than 1.8 ms., the last interval shown with this action potential still evident.

Latency change. As already shown in figures 3 and 4, there is a pronounced latency increase during the refractory state. The fast closer fiber of the crayfish shows a mean latency incease of 50.6%, attributable perhaps to a conduction velocity decrease of about 33%. The mean values for the latency increase for slow closer and opener are 54.5 and 52%, respectively. As small an increase as 13.3% and as large as 166.7% have been recorded from opener fiber preparations. Similar ranges of latency increase have been observed with the other fibers. In the lobster nerve fibers the mean values and ranges of latency change are somewhat higher, a mean of 80% for the slow closer for example. The fast closer fiber of the lobster shows a latency increase of 52% on the average and the opener fiber 70.6%.

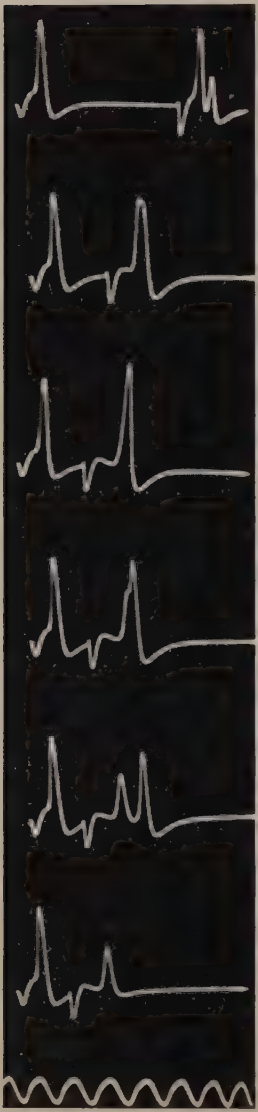

Figure 5

Good examples of the latency increase or conduction velocity decrease in lobster nerve fibers are pictured in figures 5 and 6. In figure 5, a double fiber preparation, the two stimuli were adjusted so that both were above threshold for the large fast closer fiber, but only the second one was above threshold for the smaller slow closer fiber. This was done by taking advantage of the fact already established that with very short duration shocks, these were less than 0.1 ms., the threshold of the large fiber is lowest, as shown in the crossing over of the strength-duration curves. In this experiment then as the interval between shocks is decreased, only the larger spike shows the effect of the refractory state. In this preparation, also, the length of nerve between stimulating and recording leads was such, about 5 mm, that the action potentials of the respective fibers are distinctly separated by the difference in conduction velocity, about 20 M/sec. for the fast closer fiber and 10 M/sec. for the slow closer fiber. As the second stimulus enters the refractory state produced in the fast closer fiber by its first impulse the latency for the second large spike is increased by a factor of 4. The large spike of the fast closer

Fig. 5 Lobster fast and slow closer preparation. Initial shock is weak and below slow closer fiber threshold but above fast closer threshold. Second is strong and above both thresholds. Note the marked latency increase in fast closer spike. 1000 ~, 10 mv. marker.

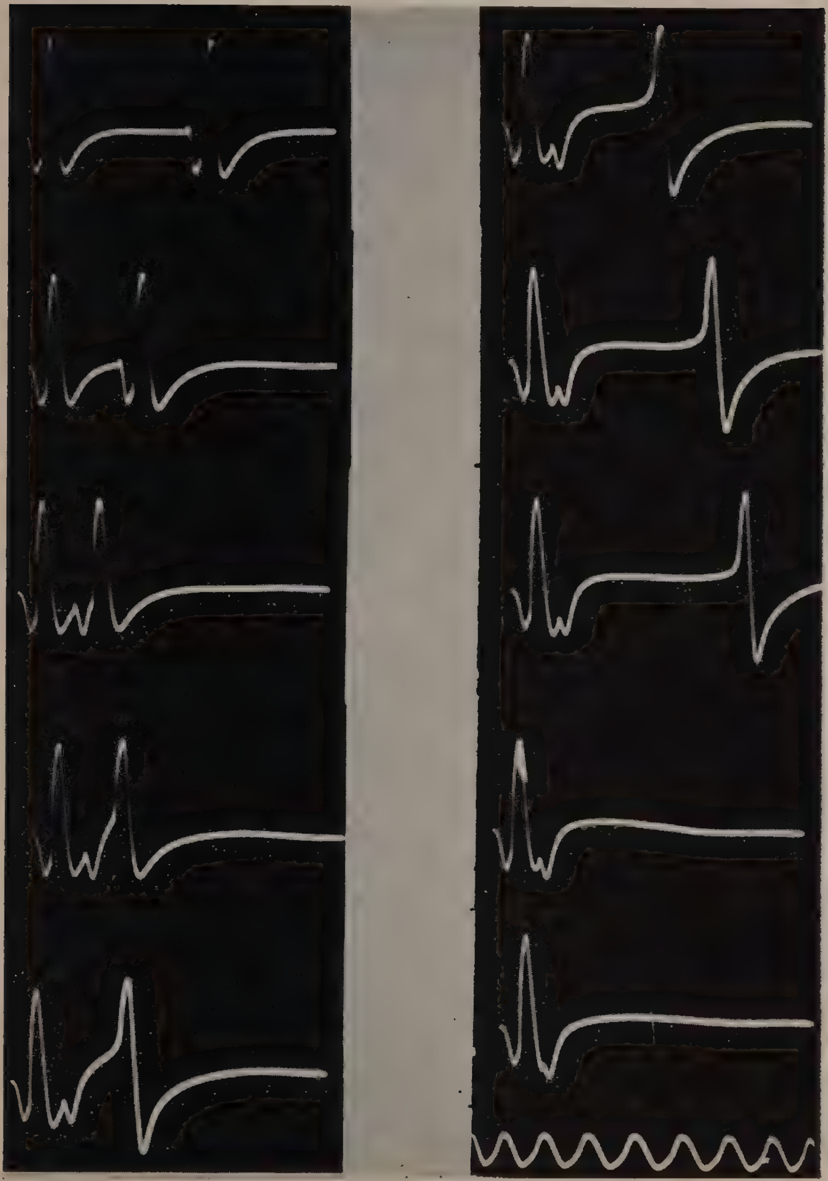

Fig. 6 This lobster slow closer fiber showed a remarkable latency increase in the refractory state. 1000 ~, 10 mv.

fiber appears to be actually "riding over" the small spike of the slow closer fiber. It is of interest to note that during the "riding over" period when the two spikes occur simultaneously (third from top) the height of the compound action potential is equal to the sum of the heights of the two action potentials when they are firing separately.

An even more dramatic example of latency increase was recorded from a lobster slow closer fiber as illustrated in figure 6. In this preparation an original latency of about .5 ms. was increased in the refractory period to 4.5 ms. In other words an original conduction velocity of 10 meters/sec. was reduced to a velocity of only 1 meter per second. There is no apparent relation between latency change and fiber type.

Spike height. Although no significant change in spike voltage is apparent in figures 5 and 6, in spite of the marked decrease in conduction velocity, some reduction in the spike height usually occurs in the refractory state. A pronounced decrease in the action potential of the slow closer fiber (fig. 4, col. 1) is clearly shown. On the average the spike height of the fast closer fiber decreases 13.2% before extinction, the slow closer, 16.7% and the opener fiber, 28.4% in the crayfish. As indicated in table 1 the decrease in spike height in the refractory state is as much as 64% in some crayfish preparations, and is not observed at all in others (note fig. 5). The decrease in spike height of lobster fibers is of the same order (table 2), ranging from 0% to as much as 90%. The changes of spike height in the refractory state (lobster opener fiber) are well illustrated in figure 7. In this experiment the stimulating and recording leads were well separated as indicated by the long latency seen in the upper picture with weak stimuli. It can be seen in this compound picture that under these conditions the second spike elicited 3.5 ms. after the first spike, is somewhat smaller than the first and is abruptly extinguished without further change in size when this interval is shortened to 3.2 ms. With stronger

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Fig. 7 Lobster opener fiber. With weak stimulus, upper picture, the spike height does not decrease as much before blocking in the refractory state as with stronger stimuli, lower records. 1000 ~, 10 mv.

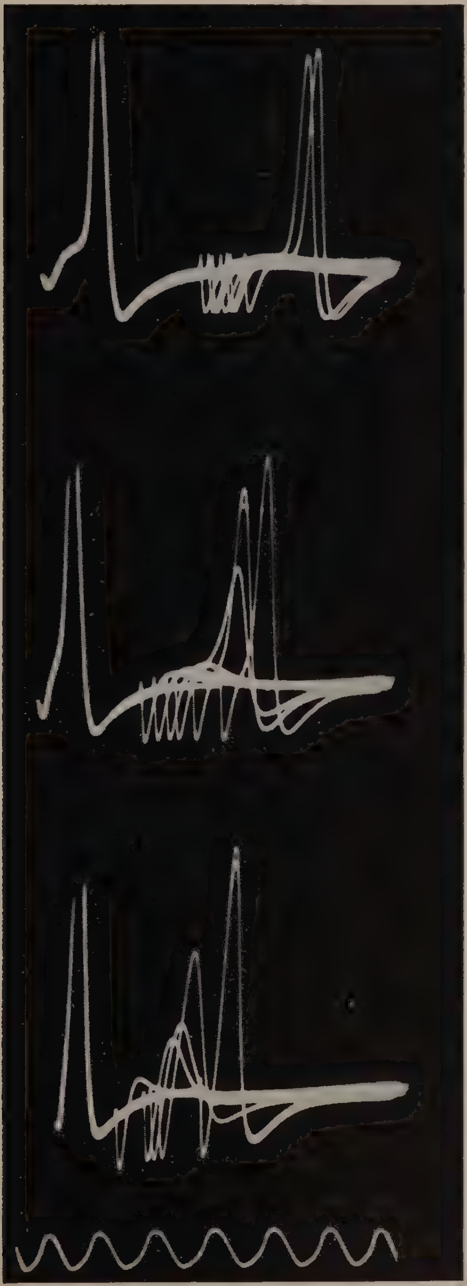

Figure 7

stimuli a more graded decrease in the second spike height is observed as the interval between stimuli is reduced. The smallest of these graded responses is about 50% normal, and can be easily distinguished from the smaller local response which becomes evident with still stronger stimuli at very short shock intervals as shown in the bottom picture of the figure.

Although the mean per cent spike height reduction differences do not appear large enough to establish any sort of relationship with specific fibers, the few results from multiple fiber preparations do suggest (table 3 and 4) that the action potential of the large fast closer fiber in crayfish and lobster can be reduced much less than the spikes of the smaller fibers and still be conducted. This is also in agreement with the mean values tabulated in table 1, but this is contrary to the mean data for lobster shown in table 2.

Repetitive discharge rate. The fast closer fiber of the crayfish does not fire repetitively during prolonged D.C. stimulation. With the same stimulation both the slow closer and opener fibers can produce a train of impulses. The average values of the maximum frequency for each fiber are 196/sec. for the slow closer, and 255/sec. for the opener. Maximum frequencies as low as 100/sec. and as high as 325/sec. were recorded for these fibers. The lobster nerve fiber preparations possess similar properties for repetitive firing except that the fast closer of this animal is also capable of firing repetitively. However, the repetitive firing of this fast closer is limited to only a few impulses as compared to the long trains of impulses which can be continually produced for the duration of the applied stimulus current by the other fibers.

A typical example of the repetitive firing pattern of the three types of fibers from the lobster is shown in figure 8. The

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Fig. 8 Lobster Triple fiber preparation. D.C. stimulation along the whole sweep. Increase in strength top to bottom records. Top record 1 slow closer spike, record 2 one fast, two slow closer spikes, one opener spike, record 3 one fast one slow spike summed, then four more slow closer fiber spikes and four opener fiber spikes interspersed; bottom record fast and slow closer fiber spikes summed, then seven slow closer fiber spikes and eight opener fiber spikes. Note response times are different for the three fibers. 60 ∼, 10 mv. timer.

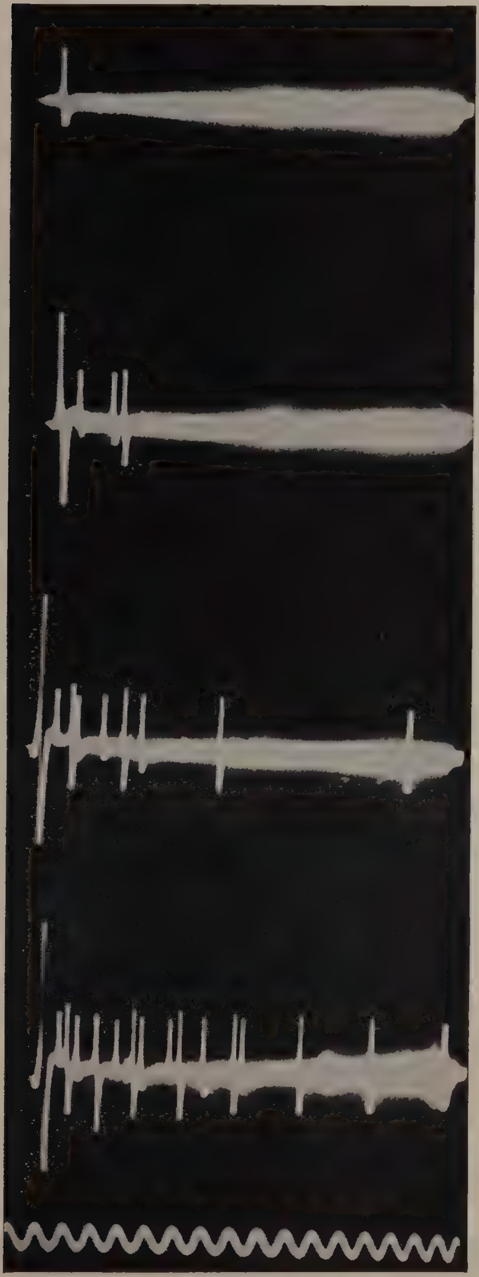


Figure 8

four records were made with increasing D.C. current applied to the preparation beginning with just threshold strength for the slow closer fiber (top picture). As the applied current strength is increased the thresholds for both the fast closer largest spike and opener fibers are attained (second picture) and two small impulses are elicited from the slow closer fiber. Further increase in the strength of the stimulus produces about the same number of repeated discharges from the slow closer and the opener fibers and an enlargement of the fast closer spike due to summation with an early slow closer action potential. As shown in these figures the oscillatory firing of the slow closer is more quickly "damped" than that of the opener fiber, although the opener fiber can discharge at as high a frequency as the slow closer. After an early high frequency burst at the beginning of stimulation, the discharge rate of the opener settles down to a lower value at which impulses may be produced for remarkably long periods of time. The maxi-

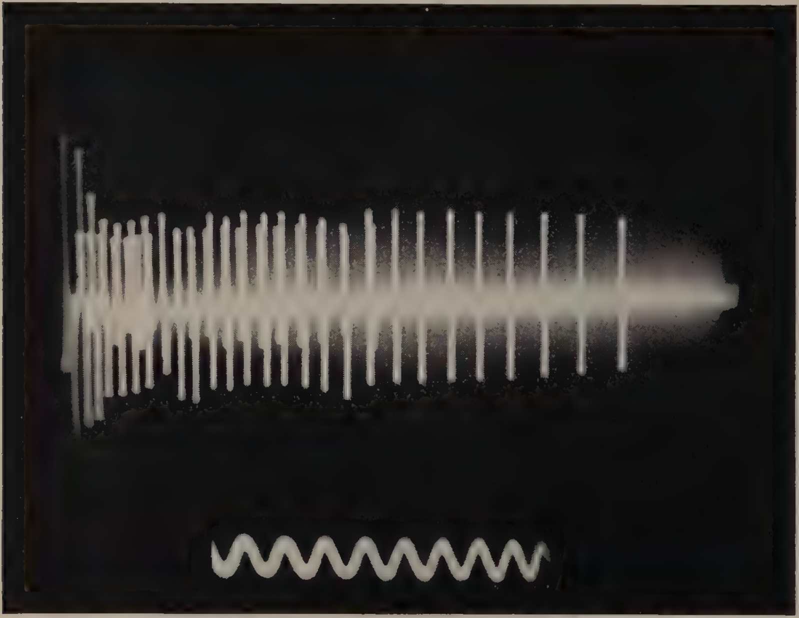


Fig. 9 The same preparation with strong stimulation. 60 ∼. 10 mv. timer. This record shows how differently the three fibers respond to the identical stimulus.

mum frequency for either fiber is not reached in this series of records, the initial frequencies being 60/sec. for the opener fiber and 80/sec. for the slower closer fiber. The slow closer fiber ceases to discharge repetitively when the frequency has dropped to 45/sec., whereas the opener fiber continued to fire at about 20/sec. for some time. A very strong D.C. pulse applied to this same preparation produced the record illustrated in figure 9. Here the initial frequencies are 300/sec. for the smaller impulses coming from the slow closer fiber, and 280/sec. for the opener fiber.

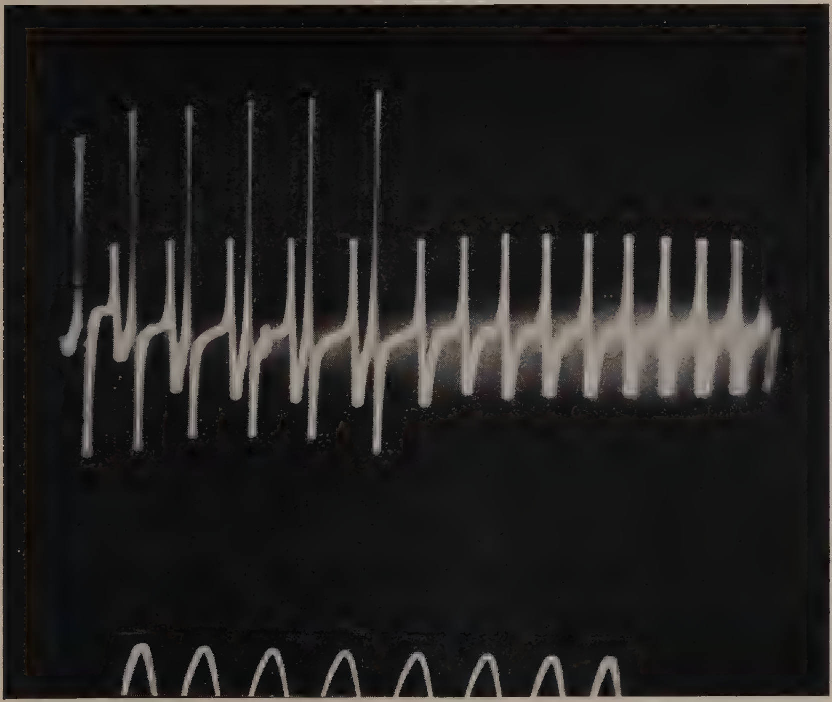


Fig. 10 Interaction of fast and slow closer fiber spikes during repetitive firing of both. 60 ~ timer. Note increased frequency of smaller spike (slow closer fiber) after larger spike (fast closer fiber) disappears.

One complication encountered in measuring frequencies of repetitive firing in these multiple preparations is presented by interaction between fibers (fig. 10). It will be noted that upon

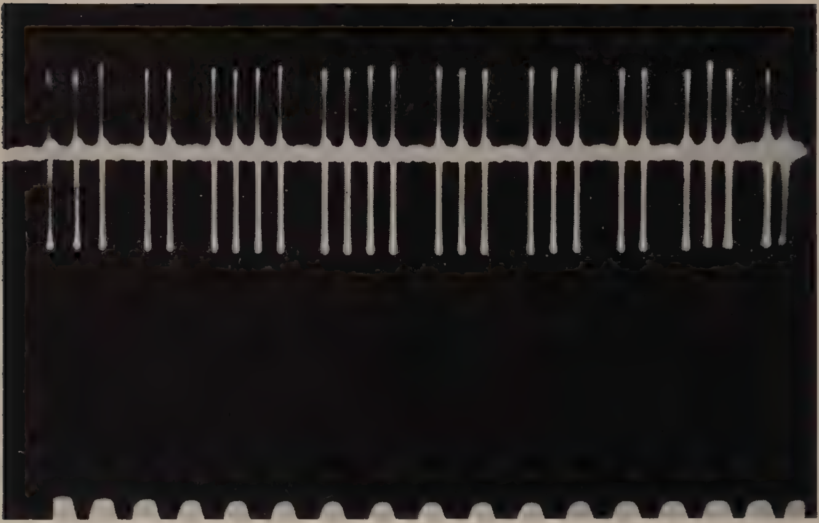


Fig. 11a Lobster opener fiber repetitive discharge. Note the "gaps" when one or more impulses have dropped out. 60 ~ timer.

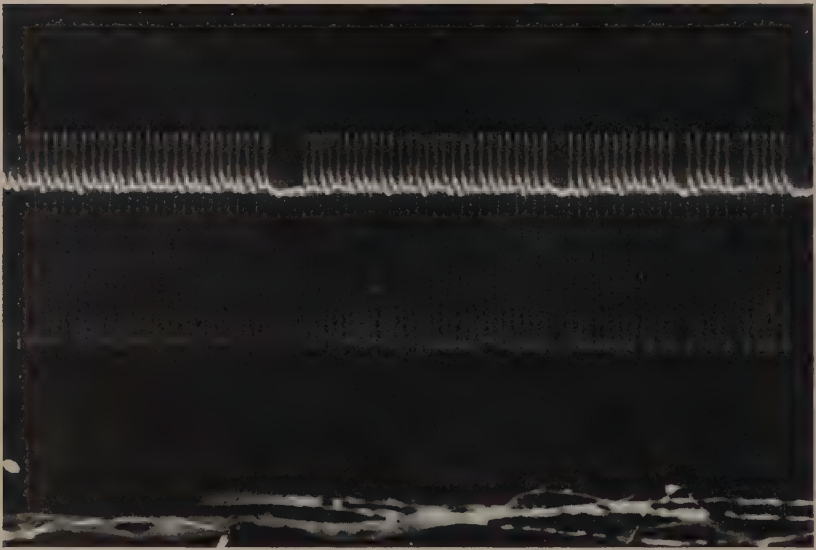


Fig. 11b Crayfish opener fiber repetitive discharges. The "gaps" are multiples of the normal interval between spikes.

cessation of the large fast closer response the smaller slow closer spikes assume a different rhythm, 180/sec., almost double that of the early spikes (100/sec.).

During a continuous train of impulses irregularities in firing often appear. Figure 11, a and b, illustrates this phenomenon in two different opener fiber preparations. The irregularities, or gaps, in the repetitive discharge record are always equal to multiples of the normal spacing between the impulses, showing that when one, 11a, or more, 11b, spikes have dropped out, the rhythm remains constant. This constancy does not prevail during the changes in spacing found in the dying out phase of a train of repetitive impulses. With high amplification a 10–20 mv. oscillatory deflection of the baseline, about one-quarter to one-tenth the size of the spike, is discernible in these "gaps," figure 12. It is obvious that this smaller response of the fiber, a lobster fast closer in this case, maintains the identical frequency as the spike, even as it "damps out" when the repetitive firing ceases. This last phenomenon as occurring in a lobster slow closer is better shown in figure 13.

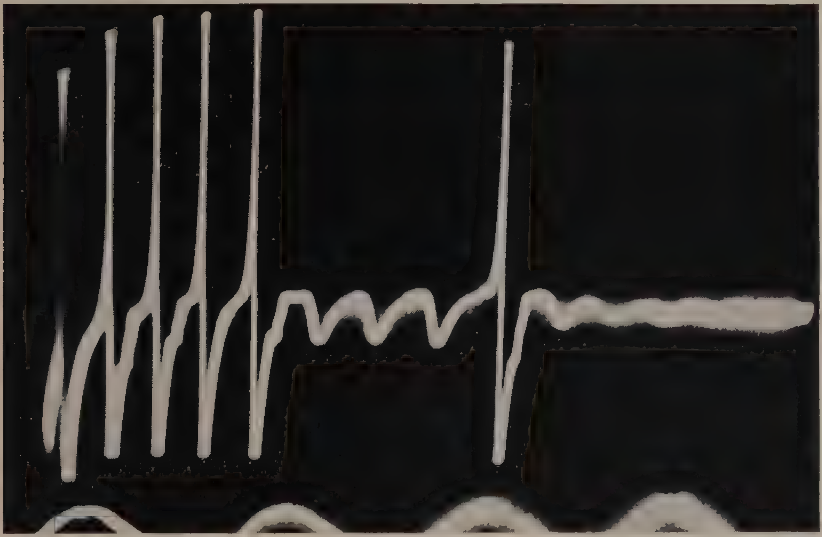
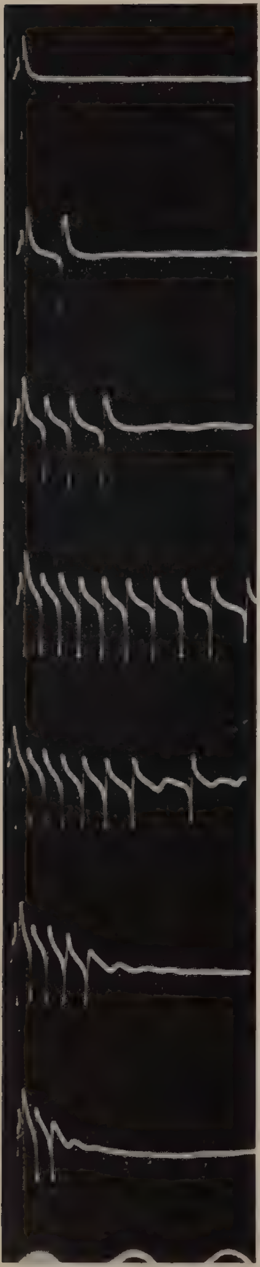


Fig. 12 Lobster fast closer fiber firing short burst with a gap at high amplification. The repetitive firing of the local response is apparent in the gap and the little responses damp out after the last spike. 60 ~ timer.



Finally, there is a very interesting comparison of lobster slow closer fiber action potentials elicited repetitively with D.C. of increasing strength and singly with two short duration shocks, figure 14. As the frequency of repetitive discharge increases with stronger stimulus (top to bottom, left side of figure), the action potentials change in shape in the identical way as does the second action potential occurring in the refractory state (right side of figure). The spike falls away from the small potential when a frequency of about 450/sec. or a separation of 2.2 ms. is attained during repetitive firing, and only the small potential remains at 500/sec. or 2.0 ms. separation. The small potential still appears repetitively at 580/sec. or at intervals of 1.7 ms., but has decreased in magnitude from about 10 mv. at 450/sec. to about 5 mv. In the refractory period records from the same fiber, the spike falls away from the initial 10 mv. potential when the interval between strong shocks is 1.8 ms., and the small response is reduced to 5 mv. when the interval is 1.4 ms. between stimuli.

DISCUSSION

It has been shown that the motor fibers innervating the muscles of the

Fig. 13 Lobster slow closer fiber firing singly then repetitively with increasing D.C. stimulus strength top to bottom. Note gaps, oscillation in gaps and damping out of oscillations. Also note reduction in size of the last spike in the train.

pincers of the crayfish and walking limb of the lobster have very different properties of excitation and conduction. The fast closer fiber possesses a high threshold, a relatively long refractory period and a large k constant. The slow closer fiber has a low threshold, the shortest refractory period and a smaller k value. The opener fiber usually has the lowest threshold, a short refractory period, and the smallest k value.

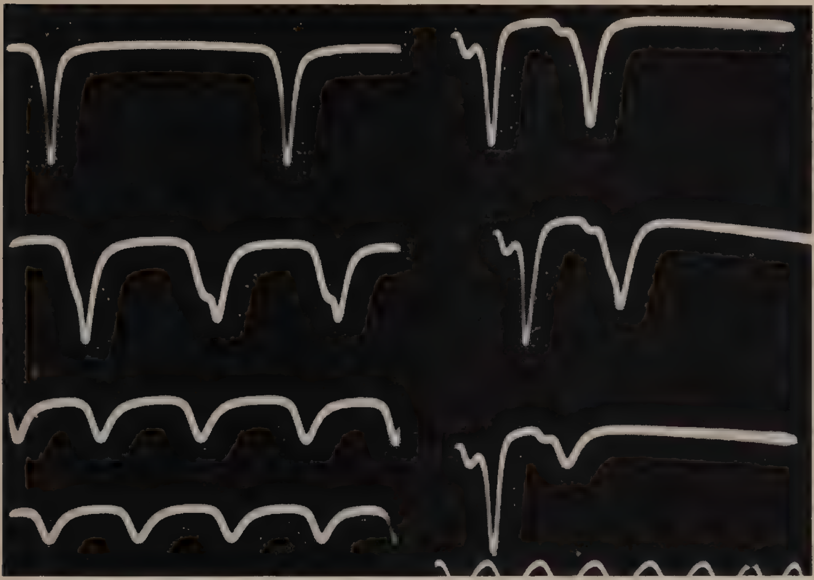


Fig. 14 Lobster slow closer fiber firing repetitively, left, with D.C. stimulation and stimulated by two shocks, the second in the refractory period right. 1000 ~ timer.

Since k is inversely proportional to t (in the equation $kt = \log_e \frac{v}{v-r}$, where t represents the time an exciting current must flow to elicit a response) k is inversely proportional to the response time. The threshold, of course, is an inverse measure of the sensitivity of the fiber, and the length of the refractory period is a measure of the time it takes the fiber to recover its excitable state again after firing. Therefore, these three fibers can be described as follows: the fast closer is relatively insensitive, slow in recovering its excitability and quick to respond.

It is logical to find that this fiber has little tendency to fire repetitively. It can be listed in group 3 of Hodgkin's ('48) classification of crustacean nerve fibers. The slow closer and opener are very sensitive, quick to recover their excitability, and have longer response times. They may be expected to respond repetitively, and are probably members of Hodgkin's group 1. Thus, as shown in figure 9, a long strong D.C. stimulus elicits one or only a few spikes from the fast closer, a brief volley from the slow closer at high frequency, and a long train of spikes from the opener fiber. This explains the triple movement response of the pincers to the mechanical stimulus of cutting through the nerve.

The smaller fibers of the animals, the slow closers and openers, are similar in their responses, but the fast closers do differ somewhat. The lobster fast closer fiber, unlike the crayfish fast closer fiber, often does respond repetitively. This may be a specie difference, but it also may be an anatomical difference, for the lobster fibers were isolated in the walking leg and the crayfish fibers in the claw. The reflex closing of the claw pincer is an abrupt twitch-like response, whereas the leg pincers reflexly close more slowly when stimulated, these two movements being best suited for the animal's survival. The sensory and central nervous system reflex mechanisms may be the same in both cases, and this response difference may be due to the differences in the electrical properties of the two fast closer fibers.

The actual mechanism of repetitive firing in the axons remains obscure, but several new facts concerning this property of the fiber have been brought to light. The small local or initial potential can "pop off" repeatedly with D.C. stimulation. The reaction responsible for this also has a refractory period. Indeed the absolute refractory periods listed above are actually the refractory periods of this local effect. As shown in figure 14 the frequency of firing of the spike and local potentials can be increased with stronger stimuli. This is because the stronger stimuli initiate the potentials further into the relative refractory state. The local impulses are probably the pacemakers of the spike repetitive discharge. This conclusion has been reached by others (Arvanitaki, '39; Brink, Bronk and Larabee, '46). In this case the maximum frequency of spike firing is limited by the refractory period of the local potential. As shown in the figure this limit is approximately 500/sec. Like the large spike, the local potential is reduced in the refractory period, so that it does not rise to threshold for triggering the spike at frequencies higher than 450/sec. if that high. Also in the refractory state the latency for spike stimulation is markedly increased which fact also affects the frequency at which a fiber can respond repetitively.

Finally the dropping out of one or more impulses producing gaps in a long train of spikes and the reduction in frequency or dying out of repetitive firing must be considered. Gaps likened to compensatory pauses when an extra response is introduced into a rhythmically firing axon have been observed by Arvanitaki ('42). There are two possible explanations. The first is based on changes in the refractory period as shown in table 5. Here it can be seen that the refractory period lengthens with time, i.e., the interval between spikes lengthens. Since the height of the local triggering response is reduced in the refractory state (also observed by Hodgkin, '38) not only will the frequency be reduced by this refractoriness, but occasionally the threshold for spike stimulation may not be reached by the local response. This would cause one or more spikes to drop out. The change in latency of response in the refractory state may also alter the frequency of discharge.

The other possible explanation is that the thresholds for both the local and propagated response may rise, i.e., accommodation may take place. If this rise is a slow one, there should be a definite period of time when the "beating" local response (shown in fig. 12) just reaches spike threshold, and occasionally does not. This would account for the uniform gaps which appear in a train of impulses and are evidently multiples of a least interval between spikes. Indeed, multiples values of a least interval of repetitive firing have been recorded from frog nerve fibers by Brink et al. ('46). The threshold for elicting repetitive local responses may also increase, damping out this oscillation. Small voltage oscillations and even threshold oscillations with a frequency of the order of 500 per second have been observed by Hodgkin ('48), Arvanitaki ('38), Shanes ('49), and LeFevre ('50), and have been recorded across the squid giant axon membrane with subthreshold stimulus by Marmont (quoted in Cole, '50). Therefore, there may be still another oscillatory membrane voltage mechanism which sets off the local response. This conclusion is in accord with the assumption by Brink et al. ('46), that nerve fibers possess a rhythmic process which maintains its rhythmic quality independently of the nerve impulses once the impulses are started.

TABLE 5

The refractory periods of lobster single motor axons measured several times after start of experiment

FIBER		MILLISECONDS			
		First	Second	Third	Fourth
Opener	1	2.0	2.2	2.3	
	2	2.2	2.8		
	3	1.6	2.6		
	4	2.4	2.6		
	5	1.2	1.8	2.2	3.0
	6	1.4	1.9		
Fast closer	1	1.5	1.8	2.0	
	2	2.6	3.0		
	3	1.8	1.8		
	4	2.4	2.8		
	5	1.8	2.0	2.6	
	6	1.8	2.0		
Slow closer	1	1.4	1.5	2.0	
	2	1.6	1.7		
	3	2.8	3.0		
	4	1.8	2.2		
	5	2.4	2.8		

The rise in threshold or accommodation (Hill, '36) now presents a problem in itself. Do these three fibers have different accommodation time constants to account for the varying durations of their repetitive volleys of impulses? This problem has been investigated and the results are presented in another paper (Wright and Adelman, '54).

SUMMARY

1. Three types of single motor fibers from the crayfish claws and lobster have been studied. It was found that these three types of fibers, each with a different function, exhibited different properties of excitation and conduction.

2. The average threshold voltages of the crayfish fibers were: .081 v. for the fast closer, .070 v. for the slow closer and .041 v. for the opener. For the lobster the threshold voltages showed the same relationship, though not as distinctly.

3. The average k constant values of the crayfish fibers were: 3.94×10^3/sec. for the fast closer, 2.82×10^3/sec. for the slow closer and 1.13×10^3/sec. for the opener. For the lobster the average k values were 2.30×10^3/sec. for the fast closer, 1.59×10^3/sec. for the slow closer, and 1.50×10^3/sec. for the opener.

4. The average refractory periods of the crayfish fibers were: 2.10 ms. for the fast closer, 1.64 ms. for the slow closer and 2.24 ms. for the opener. For the lobster the average refractory periods were: 2.46 ms. for the fast closer, 2.10 ms. for the slow closer and 2.54 ms. for the opener.

5. Measurements of decrease in spike height and increase in spike latency during the refractory period showed no marked differences among fiber types. Measurements of repetitive discharge rates showed no marked differences among fiber types except for the crayfish fast closer which could not be made to fire repetitively.

6. A possible explanation of the different properties of repetitive firing is discussed.

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THE CHANGES IN ELECTROLYTES, PARTICULARLY CHLORIDE, WHICH ACCOMPANY GROWTH IN CHICK MUSCLE [1]

J. S. BARLOW AND J. F. MANERY
Department of Biochemistry, Faculty of Medicine,
University of Toronto, Toronto, Canada

FOUR FIGURES

INTRODUCTION

A program of research on the electrolyte changes which accompany growth was initiated a few years ago in this laboratory in the hope that it might help to clarify some of the problems of ionic distribution and permeability in adult tissues. Considerable interest has also been shown recently in the appearance, or increase in concentration, of a variety of organic substances, particularly enzymes and phosphate esters, in the tissues of developing organisms. In too many instances little or no attempt has been made to relate these changes to the chemical morphology which is also altering as development proceeds. It is conceivable that the increase in some constituent may be caused merely by the replacement of extracellular water and solids by the cell type specific for the tissue being formed, and may be totally unrelated to the establishment of a new metabolic system at a functioning level.

It is generally agreed that the chloride of adult skeletal muscle is situated in the extracellular phase and that, under normal circumstances, little or none of it is to be found inside the muscle fibers. Usually, the chloride concentration of the extracellular fluid has been assumed to be that in a plasma ultrafiltrate. Utilizing this information, the volume of the extracellular water in the adult muscle has been estimated to be 10 to 20% of the weight of fresh muscle. When Barlow, Slinger and Manery ('47) found that in the breast muscle of baby chicks (three days old) there was 5 times as much chloride as in the breast muscles of chicks one month old, the problem of locating the chloride in the young muscle immediately arose. If this chloride too was confined to the extracellular phase, a decrease in the volume of extracellular water from 40% of the weight of the muscle to the adult value of 13% must have taken place during the first month after hatching. Such a decrease could occur if the chloride-rich extracellular phase were displaced during growth by chloride-poor muscle fibers. There would also appear to be a decrease in extracellular water if the muscle fibers of young organisms contained chloride which was lost from the fibers as they developed.

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[1] This research was reported in abstract in 1950 (Barlow and Manery, see references).

In the investigation to be described here two types of evidence were obtained in an attempt to resolve this problem. Firstly, the extracellular volume of the muscle calculated from the chloride content was found to be equal to the extracellular space estimated in histological preparations. Secondly, the chloride of soaked muscles was shown to exchange freely with that of the external medium over a wide range of chloride concentrations. This evidence furnished strong support for the view that in muscles of baby chicks as well as in muscles of adult organisms the chloride was typically an extracellular ion.

Acceptance of this thesis permitted a calculation of the concentration of intracellular sodium and potassium. In young muscle fibers the concentration of potassium per liter of cell water was estimated and found to be the same as that in the fibers of the adult. Analyses of the whole muscle showed an "excess" sodium (sodium in excess of that required in the volume of extracellular fluid calculated from the chloride content of the muscle). The location of this "excess" sodium will be discussed.

The data supporting these conclusions are presented under three headings: (1) the comparison of the "chemical" and "morphological" cell volumes, (2) the diffusibility of muscle chloride as demonstrated by soaking experiments, and (3) the calculated intracellular concentrations of sodium and potassium.

MATERIAL AND METHODS

Material. The young chickens used for these experiments were hatched from White Leghorn eggs. After hatching, the chicks were transferred to an incubator maintained at 37°C. In some of the earlier experiments they were given neither food nor water but later, for reasons described in "Results," distilled water was supplied ad libitum until the chicks were killed at three to 4 days of age. The birds were decapitated and, when a sample of serum was required, blood was collected in 15 ml centrifuge tubes. After clotting had occurred the blood was centrifuged and samples of serum were taken.

Because it was essential to keep the muscle viable as long as possible for these experiments, great care was taken to avoid injury during its dissection. Immediately after the chick had been decapitated and bled, the skin and fascia overlying the breast muscle were quickly lifted off and the breast muscle was removed using blunt dissection wherever possible. The muscle was first freed along its lateral boundary from its insertion at the wing to the posterior end of the keel. With a sharp scalpel the muscle was cut from the very fragile furcula, and the back of the blade was used to free it from the keel bone. The muscles were used directly for analysis, or soaked in artificial media as the experiment directed.

The "chemical" and "morphological" methods of estimating cell volume. The serum from each chick and one breast muscle were used for the determination of chloride, and from these analyses the volume of extracellular water, $(H_2O)_E^{Cl}$, was calculated (see "Calculations") and subtracted from the total water to give an estimate of the volume of cell water, $(H_2O)_C$. This provides the "chemical" estimate of the cell volume.

In order to estimate the cell volume by a "morphological" method histological sections of the contralateral breast muscles were prepared. The muscle was fixed overnight in Allen's modification of Bouin's fixative and embedded in Fisher's Tissuemat (M.P. 60°–62°C.). Ribbons containing 4–6 sections 7.5 to 10 μ thick were cut at right angles to the long axis of the muscle and placed on slides. This operation was repeated at more or less regular intervals throughout the length of the muscle so that 6–10 slides each bearing 4–6 sections represented each muscle (see fig. 1 a and b). These sections were stained with hematoxylin and eosin, and were mounted in celloidin under cover slips according to the method used by Truax ('39). The section from each slide which showed the least fragmentation when examined macroscopically was projected on a plane surface by means of an instrument which utilized parts of an inverted microscope in a fashion suggested by Dr. D. Hamly. By means of a Howard disc inserted in the ocular of the microscope which formed a part of this apparatus a grid was superimposed on the image. This grid facilitated choosing the areas for measurement. As shown in figure 1 c, each area consisted of a strip across the section at right angles to its long axis.

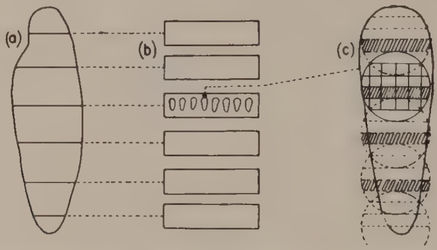

Fig. 1 Diagrams illustrating the method of measuring the morphological cell volume. Part (a) represents an entire breast muscle, the horizontal lines indicating the loci along its length at which 4 to 6 serial cross sections were taken. Part (b) shows 6 slides each with the sections from one locus mounted. Part (c) is a diagram of the section from each slide which was measured. This was done by dividing the section into 20 strips at right angles to the long axis of the section and in every 4th strip (shaded) the total and cell areas were measured.

A planimeter was adapted to measure the projected cell areas. The adaptation consisted of a new tracing pointer which was extended along the line of the arm of the planimeter (see fig. 2). This extension prevented the image at the end of the pointer from being obscured by the shadow of the finger grip of the planimeter or the hand of the operator. Extending the planimeter arm in this manner changes the calibration of the instrument, but as long as the extension is parallel to the axis of rotation of the measuring wheel, the instrument may be used to measure relative areas without restandardizing. If the extension is not parallel to the axis of rotation of the measuring wheel, readings on a standard area will vary as the relationship of the tracing pointer to the pole piece is altered.

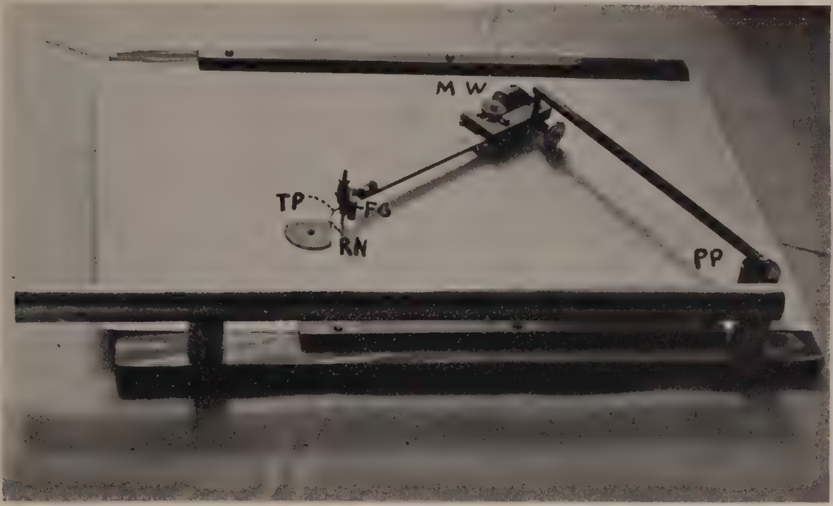

Fig. 2 A photograph of the planimeter with the altered tracing pointer and the movable platform used for measuring the total and cell areas of the projections of the histological sections. The tracing pointer, TP, is extended so that the image under the point will not be obscured by the finger grip, FG, or the hand of the operator. The extension of the pointer is parallel to the axis of the measuring wheel, MW. The planimeter is shown mounted on the platform with the planimeter pole piece, PP, in the lower right hand corner and the tracing pointer in the reference notch, RN.

The planimeter was mounted on a smooth board covered with a sheet of white paper. A piece of fiberboard notched to serve as a reference point for the planimeter tracing pointer was mounted on the board. The planimeter was placed with the tracing pointer in the notch as shown in figure 2 and an initial reading was taken. Sliding the board under the projected image of the section until the notch and pointer lay on the circumference of the particular area to be measured, the tracing pointer was moved around the outline of the area until it returned to the reference point. If, as was generally the case, a number of individual areas were to be summed, the board was now moved until the pointer in the reference notch lay on the circumference of the next area to be measured and the process was repeated. When all the desired areas had been measured in this manner, the final reading of the planimeter was taken, and the initial reading was subtracted leaving the total of all the areas traced.

In the above manner, the total area of the projection and the area of the cells in the projection were measured, and it was assumed that the ratio of the cell area to the total area of the section was equal to the ratio of the cell volume to the total volume of the muscle.

To test the reproducibility of these measurements, the ratio of the cell area to the total area of one field was determined 10 times. The results ranged from 47.4 to 51.4% cells with a mean of 48.5% and a root mean square deviation from this mean of 1.1%. Triplicate measurements on as nearly as possible the same field but 6 sections removed from the above differed by 2.3% (46.2%) and therefore it was thought necessary to measure only one section from each slide. Further study showed that the measurement of only 25% of the total section provided a fair representation of the whole. The method of choosing every 4th area for measurement with the aid of a Howard disc inserted in the ocular of the microscope is shown in figure 1 c. Since 34–60 areas were measured for each muscle, the measurements were not duplicated.

In this manner then the "morphological" estimate of the cell volume was obtained.

The feasibility of comparing the right and left breast muscles of one bird was suggested by the observation of Eggleton, Eggleton and Hamilton ('37) that chloride concentrations of paired muscles from one frog were similar. To assure the identity of paired muscles of baby chicks, the right and left breast muscles of 6 individuals were analyzed. The average difference in the chloride concentration found was 3.1 mM/kg of muscle, or about 4.5%.

Method of determining the diffusibility of chloride. In order to study the relationship between the chloride content of the muscle and the chloride content of its environment, muscles were soaked in Ringer-Dale solutions[2] modified by replacing varying amounts of NaCl with $NaNO_3$. Muscles were also soaked in a hypertonic solution (0.473 osmolar) containing one and one-half times the NaCl of the basic solution, and in a hypotonic solution (0.191 osmolar) formed by diluting the basic solution (0.328 osmolar) with an equal volume of dilute sucrose solution. The muscles were contractile after 40 minutes' soaking in all of these solutions, a period of time which was found to be sufficient to allow the chloride of the tissue to reach equilibrium with the chloride of the medium.

After dissection, each muscle was weighed on a torsion balance and transferred immediately to a 25 ml Erlenmeyer flask containing 10 ml of the desired solution. The flasks were kept in a constant temperature bath at 37°C. and a stream of warm, hydrated air or oxygen was bubbled gently through the solution to provide oxygen and to keep the contents of the flasks agitated. No significant difference could be observed between muscles which had been aerated with pure oxygen or with air for the 40 minute soaking period. The weights of the muscles were again recorded after soaking, and chloride determinations were carried out on the muscles and on the fluids. Each muscle was tested for contractility after soaking.

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[2] The basic solution contained 0.150 M NaCl, 0.00295 M KCl, 0.0004 M $CaCl_2$, 0.00003 M $MgSO_4$, 0.00173 M KH_2PO_4, 0.0116 M Na_2HPO_4, and 0.0056 M Glucose per liter.

Chemical analyses. To determine water, chloride, sodium and potassium on one sample of muscle it was necessary to pool the tissue from three or four chicks. Equal samples from the sera of all chicks were taken and mixed in a weighing bottle. The volume of the pool usually amounted to approximately 1 ml. From this, 0.2 ml were transferred to a second weighing bottle for chloride analyses; the remaining serum was used for the determination of sodium and potassium. The muscles from these chicks were pooled in a weighing bottle, and chopped finely in a hydrated atmosphere. The pool amounted to about 1 gm of muscle from which approximately 0.2 gm were transferred to a second weighing bottle for chloride analysis, and the rest used for sodium and potassium analyses.

Tissues of the adult hens analyzed were handled in a similar manner. Duplicate pieces of tissue were taken from different parts of the muscle for each analysis. Approximately 1 gm of muscle was used for chloride and 3 gm for sodium and potassium analyses.

The *water* content of the tissue was determined by drying to constant weight in a thermostatically controlled oven at a temperature of 100°C. When the complete analysis was carried out both the sample to be used for chloride and the sample to be used for sodium and potassium analyses were dried, and the duplicate results were averaged.

The *chloride* method used was essentially that of Van Slyke ('23) with the modifications suggested by Wilson and Ball ('28), but was further adapted to apply to 0.1 gm of muscle and 0.05 gm of serum. The results of 5 analyses performed on the same muscle mince by this method ranged from 52.5 to 55.7 (av. 54.1) m. eq. per kilogram and the results of 4, performed in the presence of added NaCl, ranged from 53.2 to 54.2 (av. 53.8) m. eq. per kilogram.

For *sodium* and *potassium* analyses the dried tissue was transferred from the weighing bottles to platinum crucibles with the aid of hot, glass distilled water. The resulting material was dried and charred on a hot plate in the presence of sulphuric acid, and was ashed in a muffle furnace at a temperature not exceeding 550°C.

Potassium was determined on aliquots of solutions of the ash by the method of Shohl and Bennett ('28) with the modifications described by Fenn et al. ('38). The method was further altered to estimate 0.005 mM of potassium. By this method standard solutions could be analyzed within 2%. The average difference between duplicate analyses on frog muscle was 2% (12 pairs), and known amounts of potassium added to rat muscle or serum were recovered within ± 1% of the added amount.

Sodium analyses were performed by a semi-micro modification of the technique of Butler and Tuthill ('31) which required 0.05 m. eq. of sodium and so necessitated the pooling of muscle from three or four chicks. Before this method was adopted three micro-chemical methods were critically tested. These were published by Woelfel ('38), Clark et al. ('42) and Lowry ('46). None proved to be as satisfactory as the gravimetric method adopted, the details of which are as follows. Phosphates were removed from solutions of the ash by the addition of calcium hydroxide followed by filtration. An aliquot of the filtrate containing approximately 0.5 m. eq. of sodium was transferred to a tared centrifuge tube and dried. The residue was dissolved in 6 drops of 1 N HCl, 8 ml of the zinc uranyl acetate precipitating reagent were added and the mixture was stirred mechanically for 8 minutes. The tube was capped and allowed to stand for one hour to complete precipitation, then centrifuged and the supernatant removed. The precipitate was washed once with glacial acetic acid saturated with the triple salt, and once with dry ethyl ether. The tubes were then dried for one hour in a drying oven at 100–103°C., cooled and weighed. Standard solutions of NaCl were analyzed by this method with an accuracy of ± 2%, and it was possible to recover known amounts of sodium added to muscle with the same degree of accuracy.

CALCULATIONS

The system of calculations adopted is similar to that used by Manery and Hastings ('39) except that the distribution ratios for diffusible ions differ slightly. These calculations are based on the usual assumptions: (1) that all the chloride of the tissue is present in the extracellular phase, (2) that the concentrations of the ions in the extracellular fluid may be calculated from their concentrations in serum by utilizing certain electrolyte distribution ratios, and (3) that the sodium and potassium not accounted for in the extracellular fluid on the basis of the first two assumptions is inside the cells. The distribution ratios which have been used, 0.977 for chloride, 0.942 for sodium, and 0.742 [3] for potassium are averages of those collected from the literature pertaining to *in vivo* fluids (Manery, unpublished). Throughout the calculations, the subscripts S, E, C and T represent serum, extracellular phase, cells and whole tissue respectively; square brackets represent milliequivalents or gram per kilogram of water, and parentheses represent milliequivalents or gram per kilogram of tissue, therefore:

$$[\mathrm{Cl}]_E = \frac{[\mathrm{Cl}]_S}{0.977}$$

$$[\mathrm{Na}]_E = [\mathrm{Na}]_S \times 0.942$$

$$[\mathrm{K}]_E = [\mathrm{K}]_S \times 0.742.^3$$

The extracellular water can be calculated as follows:

$$\frac{(\mathrm{Cl})_T}{[\mathrm{Cl}]_E} \times 1000 = (\mathrm{H_2O})_E^{\mathrm{Cl}} \text{ (gm/kg of tissue)}$$

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[3] Since the preparation of this manuscript it appears that a distribution ratio for potassium of 0.943 is more accurate for *in vivo* fluids than 0.742 (see Manery, Physiol. Rev., in press). Although the absolute value of $[\mathrm{K}]_C$ for the young chick (table 3) is changed slightly if 0.943 is used, no conclusions are affected.

and the cell water is given by the expression,

$$(H_2O)_T - (H_2O)_{E}^{Cl} = (H_2O)_C \text{ (gm/kg of tissue).}$$

The amounts of sodium and potassium in the extracellular water can be calculated by multiplying their concentrations in the extracellular water by the volume of the extracellular water.

Thus $\frac{[Na]_E}{1000} \times (H_2O)_{E}^{Cl} = (Na)_E$ (m. eq./kg of tissue)

and $\frac{[K]_E}{1000} \times (H_2O)_{E}^{Cl} = (K)_E$ (m. eq./kg of tissue).

Subtracting these values from the totals for the tissue gives the quantities which are assumed to be in the cells.

$$(Na)_T - (Na)_E = (Na)_C \text{ (m. eq./kg of tissue)}$$
$$(K)_T - (K)_E = (K)_C \text{ (m. eq./kg of tissue).}$$

The concentrations of these may then be calculated per kilogram of cell water.

$$\frac{(Na)_C}{(H_2O)_C} \times 1000 = [Na]_C \text{ (m. eq./kg of cell water)}$$
$$\frac{(K)_C}{(H_2O)_C} \times 1000 = [K]_C \text{ (m. eq./kg of cell water).}$$

RESULTS

1. *Comparison of the "chemical" and "morphological" cell volumes.* The extracellular position of chloride of muscle was indicated some years ago by the similarity between the extracellular volume calculated from the chloride concentrations in frog muscle and serum by Fenn, Cobb and Marsh ('34), and the estimate of the extracellular volume of frog muscle made from measurements of frozen sections by Hermann (1888). Truax ('39) later showed that the extracellular volume of liver as calculated from chloride analyses agreed well with an estimate obtained by measurement of the extracellular spaces in histological sections. He weighed photomicrographs of liver sections, cut out the cells and weighed the remainder, thus finding the proportion of cells to extracellular space. A similar comparison was made by Truax and Manery (unpublished) between the chloride

space and the histological extracellular volume of the muscles of normal and scorbutic guinea pigs. In liver and in normal and scorbutic guinea pigs a close correspondence between the two measurements was found.

In column 4 of table 1 the final estimate of the morphological cell volume of each chick muscle is recorded; these figures are obtained by direct measurement, with a planimeter, of the total area and the cell area in histological sections according to the method already outlined. The cell area of a section is expressed as a percentage of the total area, and the cell volume of the entire muscle is considered to bear the same relation to the total volume as the cell area to the total area. To obtain the figures of column 5 the volume of extracellular water, $(H_2O)_E^{Cl}$, calculated from chloride analyses was subtracted from the total water of the muscle and

TABLE 1

Comparison of the results of "chemical" and "morphological" estimation of cell volume

CHICK NO.	NO. OF SECTIONS MEASURED	NO. OF AREAS MEASURED	ESTIMATED CELL VOLUME [1] PER KG OF MUSCLE		DIFFERENCE (Chem.-Morph.)
			Morphological	Chemical	
(1)	(2)	(3)	(4)	(5)	(6)
1	9	55	428	479	+ 51
2	9	48	473	458	— 15
3	9	51	421	478	+ 57
4	10	60	436	412	— 24
5	6	34	466	466	0
6	10	56	361	339	— 22
7	6	41	415	466	+ 51
8	9	43	407	432	+ 25
9	10	52	433	426	— 7
				Average	+ 13 ± 31

[1] Assume specific gravity = 1.

"Chemical" estimate of cell volume $1000 - (H_2O)_E^{Cl}$.

"Morphological" estimate of cell volume $\frac{\text{area of cells}}{\text{total area of section}} \times 1000$.

the resultant cell volume expressed as a percentage of the total volume.

The close correspondence of the values obtained from the two methods of estimation is evident from the data of column 6, the maximum difference being 5.7% of the total muscle volume, and the average difference + 1.3% with a standard deviation of ± 3.1%. When it is realized that the chloride concentrations of right and left muscles differ by about 5%, the conclusion is amply justified that the chemical and morphological cell volumes are equal within this limit. This supports the view that although the chloride concentration of chick muscle is much greater than that of the adult muscle, most of it must be in the extracellular phase in a concentration similar to the plasma chloride concentration. Even a cursory examination of the histological sections showed the well rounded muscle fibers widely separated. It is of some interest, too, that the myofibrils within each fiber were likewise well separated and a clear core which seemed to lack fibrils was observed in the center of each fiber.

2. The diffusibility of chloride and the osmotic behavior of muscle. If the large quantity of chloride found in young chick muscles is situated in the extracellular phase as the data in the preceding section indicate, the chloride of soaked muscle should diffuse freely into the external medium. A linear relation should exist between the concentration of chloride in the soaking fluid and that in the muscle. This was found to be the case for the muscle chloride of the frog (Fenn et al., '34; Eggleton et al., '37) and of the cat (Amberson et al., '28; and Yannet and Darrow, '40). In the research reported here it has been shown to be the case for soaked breast muscles of the three day old chick as figure 4 demonstrates. Obviously this soaking procedure does not permit a conclusion regarding the permeability of the muscle fiber to chloride, nor is it sufficiently precise to determine that a small amount of non-diffusible chloride does not exist in the tissue. Yannet and Darrow ('40), for example, postulated that in cat skeletal muscle 1 mM of chloride per

TABLE 2

Chloride and water in breast muscle, freshly dissected or soaked in a variety of salt solutions
All muscle concentrations are based on initial wet weights (Wi)

EXP. (1)	SOAKING FLUID			MUSCLE				
	Type (2)	Conc. osmol. (3)	Cl m.eq./l (4)	Cl m.eq./kg (5)	$(H_2O)^{Cl}_{E}$ ml/kg (6)	Solids % Wi (7)	Wt. gain gm/kg Wi (8)	$\Delta(H_2O)^{Cl}_{E}$ (9)
I (14)	serum		122.6	69.4	511	16.4		
	Ringer	0.328	153.4	101.6	664	15.2	+ 2	+ 153
II (5)	serum		122.2	81.1	606	14.8		
	Ringer sucr.	0.236	102.2	64.1	626	13.1	— 63	+ 20
III (5)	*20 min.*							
	serum		118.5	52.8	408	21.5		
	Ringer sucr.	0.191	77.7	44.6	575	19.8	+ 123	+ 167
(5)	*40 min.*							
	serum		127.4	65.0	468	19.7		
	Ringer sucr.	0.191	77.6	51.0	655	17.8	+ 124	+ 187
(5)	*60 min.*							
	serum		125.7	60.8	445	22.4		
	Ringer sucr.	0.191	76.5	52.7	690	19.9	+ 222	+ 245
IV (4)	serum		130.1	70.1	485	18.5		
	Ringer sucr.	0.145	52.1	34.8	668	18.7	+ 95	+ 183
V (4)	Ringer	0.328	152.5	102.0	675		+ 30	
	Ringer NO_3	0.328	3.8	6.2	. . .		+ 34	
(5)	Ringer	0.328	156.4	106.8	680		+ 69	
	Ringer 1/2 NO_3	0.328	82.9	57.4	692		+ 110	+ 12
VI (6)	Ringer	0.328	152.8	103.3	678	16.5	+ 95	
	Ringer sucr.	0.191	80.6	51.3	635	16.6	+ 127	— 43
VII (6)	Ringer sucr.	0.237	121.9	77.2	634		+ 88	
	Ringer sucr.	0.203	86.5	54.9	634		+ 99	0
VIII (5)	Ringer	0.328	150.9	105.5	700	14.2	+ 35	
	Ringer NaCl	0.473	226.5	166.4	734	14.6	+ 29	+ 34

Forty minutes was the soaking period unless otherwise stated. Bracketed numbers in column (1) represent the number of pairs of muscles contributing to the average value quoted. Values in colum (6) for all soaked muscles are obtained as follows (col. [5] ÷ col. 4) × 1000, while those for muscles in equilibrium with serum involve the use of the distribution ratio (see "calculations").

100 gm of fat free solids will not diffuse freely into the environmental solution. Boyle et al. ('41) and Boyle and Conway ('41-'42) estimated that frog muscle fibers *in vivo* contained chloride amounting to about 1% of the external concentration.

A detailed discussion of the data shown in figure 4 and table 2 ensues.

Early in this research an attempt was made to deplete the muscle chloride by intraperitoneal injection, but the variation owing to the different growth rates found in individuals of the same age was so great that the method had to be abandoned. In lieu of this procedure, *in vitro* soaking experiments were carried out permitting a comparison between the right and left breast muscles of each individual. The chicks used for these data were given water *ad libitum* after hatching (see Results, part 3). All soaked muscles were contractile at the end of the soaking period unless otherwise stated.

The data have been calculated in terms of initial weights rather than final weights. The choice depends on the information desired. Muscles gain weight on soaking in artificial salt solutions. Hence the initial weight is used as the basis of calculation when the changes occurring in members of a pair are being compared. When the final composition of the muscle is being studied the final weight is used as the reference point. Since the weight gain is given in column 8 of table 2 any figure may be recalculated on the basis of the final weight.

(a) Serum versus salt solutions. In the first 4 experiments one member of a muscle pair was soaked in an artificial salt solution of the osmolar concentration indicated in the table, and its composition compared with that of the freshly dissected contralateral muscle. The latter was considered to be in equilibrium with serum. The chloride concentration (table 2, col. 5) of these normal muscles ranged from 53 to 81 m. eq. per kilogram illustrating the variation which occurs within an age group; the calculated extracellular water

(col. 6) was likewise variable. Even in isotonic Ringer's solution soaked chick muscles usually gained weight. This is more clearly illustrated in experiments V to VIII (col. 8) inclusive than in I to IV, partly owing to the technical difficulty of establishing a consistent blotting procedure to obtain the initial weight.

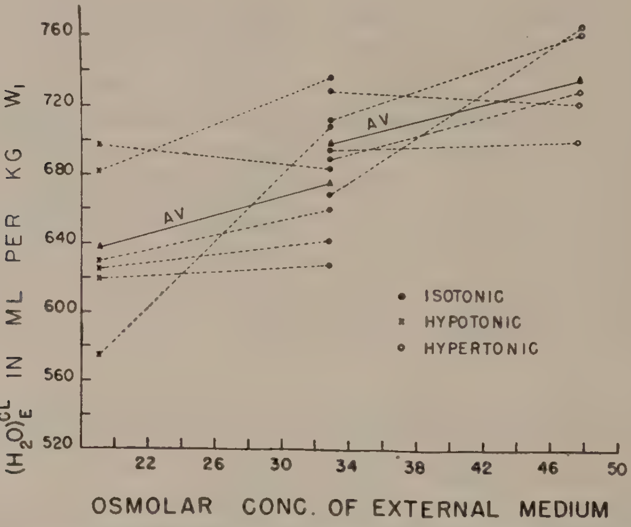

Fig. 3 The influence of the osmotic pressure of the external medium which is a balanced salt solution on the calculated extracellular volume, $(H_2O)^{Cl}_{E}$, of chick breast muscle. Dotted lines join matched pairs. Solid lines represent the average change in the 5 or 6 pairs of muscles used.

Although the variation in the data was large, in general, the increase in weight during soaking was greatest in media of the lowest osmolar concentration and much of this weight increase was undoubtedly extracellular water. Pertinent, too, to this change in weight and probably contributing to the variability of the data is the loss of solids (average 10 gm per kilogram) which occurred frequently during the soaking period. Micro-Kjeldahl analyses of the immersion fluid confirmed a similar finding of Fenn's ('35) that muscles soaked in Ringer's solution lost nitrogen.

In experiment III the muscles were soaked for different periods of time. Obviously most of the changes had taken place in 20 minutes. Because one of the muscles was not contractile after soaking for 60 minutes and because the average gain in weight and in extracellular water (cols. 8 and 9) was considerably larger than in muscles soaked for shorter periods, it was decided to adopt a 40 minute period routinely. In experiment IV 4 muscles were soaked in the most hypotonic Ringer's solution (0.145 osmolar) for 15, 25, 35 and 45 minutes, respectively. Normal contractility was lost in 25 minutes but there were no significant differences in the chloride concentrations which could be attributed to the period of soaking. Again there was evidence that equilibrium was reached in about 15 minutes.

(b) Ringer's solutions versus modified Ringer's solutions. Because of the changes which invariably occur on dissection and soaking, in all subsequent experiments both members of a muscle pair (table 2, V to VIII inclusive) were soaked in artificial salt solutions. The experimental muscle in a modified salt solution was compared with the contralateral control which had been in normal Ringer-Dale for the same length of time.

In experiment V in which isotonic low-chloride solutions were prepared by partially or completely replacing the chloride by nitrate, the experimental muscles lost chloride and gained weight but the volume of extracellular water was the same in the muscles soaked in Ringer-1/2 nitrate as in normal Ringer's solution. The muscles in the solution containing only about 3.8 m. eq. of chloride per liter lost almost all of the chloride which they had originally contained. The amount retained was too small for accurate analysis so that no significance can be attached to the fact that the calculated extracellular water would exceed 1000 ml.

In the last three experiments the osmotic behavior of the muscles was studied. Muscles in hypotonic (VI) and hypertonic (VIII) solutions were compared with their mates in normal Ringer's solution. The individual sets of data (with one exception) as well as the average values showed that the muscles in hypotonic media had less extracellular water (col. 6), and in hypertonic media more extracellular water, than their controls in isotonic solutions (see fig. 3). This situation would arise if the muscle fibers swelled in hypotonic solutions and displaced extracellular fluid, and conversely shrank in hypertonic media with the resultant enlargement of the extracellular space. Similar findings and interpretations have been reported for soaked frog muscle (Overton, '02; Fenn, '36a; and Steinbach, '44). That variations in tonicity should produce changes in the volume of the cell phase is to be expected but failure of the chick muscle as a whole to gain or lose weight on exposure to hypo- or hypertonic solutions, respectively, is less easily explained. All muscles in artificial salt solutions swelled regardless of the tonicity of the soaking medium.

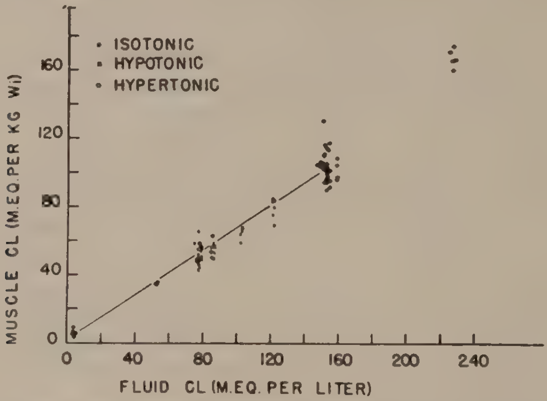

Fig. 4 Data showing a linear relation between the chloride content of soaked breast muscle and that of the external medium. All data contibuting to the averages in table 2, columns 4 and 5 are represented. The straight line is drawn through the averages for isotonic solutions of varying chloride concentrations.

It is not possible at this time to interpret the uptake of water by isolated chick muscles in isotonic and hypertonic solutions. Any entrance of potassium chloride into either normal or damaged muscle fibers would result in an uptake of water and an apparent increase in $(H_2O)_E^{Cl}$. Conway and collaborators ('45, '46, 50) have interpreted in this way the swelling which occurs in isolated frog sartorii when potassium replaces sodium. In our experiments the potassium concentration was always low and the potassium: sodium ratio remained unaltered except in experiment VIII when the ratio was decreased by the addition of sodium chloride to produce a hypertonic solution. Without potassium analyses we cannot evaluate this mechanism. Attention is drawn, however, to the large extracellular space in these muscles which, judging from the gelatinous nature of the tissue, appears to contain organic colloids. If they imbibe water due to a colloidal osmotic pressure not shared by the soaking medium a consistent increase in weight is to be expected. Injured fibers would also contribute to the colloid osmotic pressure. The uptake of water by excised frog muscles in isotonic and hypertonic salt solutions is still not clearly understood (Wolf, '40; Chao, '39).

Regardless of the exact location of the chloride in the isolated chick muscle there is no doubt about the fact that the tissue chloride bore a simple linear relationship to the chloride concentration of the soaking medium. In figure 4 are plotted all of the analytical data contributing to the averages listed in table 2. The line is drawn through the averages of the three sets of data obtained from tissues immersed in isotonic media. The points representing tissues in hypotonic media are slightly below the line while those from muscles in hypertonic media are above it. The slope of the line is 0.62 which, if all the chloride is extracellular, is interpreted to mean that 62% of the soaked muscle is extracellular space.

3. Distribution of sodium, potassium, chloride and water in chick muscles. For the data presented in a previous publication (Barlow et al., '47) and for some to be reported here the chicks were kept in an incubator without water from the time of hatching to the experimental period. In both of these sets of data great variation was observed among individuals. To ascertain the extent to which deprivation of water was responsible for the variability, 13 chicks were placed in an incubator and given water *ad libitum* for three days after hatching, and the muscles and sera analyzed for chloride and water. The average muscle and serum water concentrations were 844 ± 11 (standard deviation) and 943 ± 5 gm per kilogram and the average chloride concentrations were 72.9 ± 5.1 and 127.9 ± 3.6 m. eq. per kilogram for muscle and serum,

TABLE 3

Serum and muscle electrolytes of young and adult animals

A. Original data

ANIMAL	SERUM				SKELETAL MUSCLE			
	H_2O	Cl	Na	K	H_2O	Cl	Na	K
	gm/kg	m.eq./kg H_2O			gm/kg	m.eq./kg wet wt.		
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Chicken, 3–4 days	937	129.8	158.6	7.0	827	68.2	96.6	45.5
	± 4	± 7.4	± 7.6	± 1.2	± 16	± 7.3	± 7.2	± 4.9
Adult	938	130.5	162.5	7.4	741	9.3	16.4	98.3
Frog (1)	960	74.3	103.8	2.5	800	10.9	25.4	83.0
Rat, 15 days (2)	938	94.2			797	31.7		
Adult (3)		113.0 [1]	146.9 [1]	4.0 [1]	773 [2]	16.3 [2]	22.7 [2]	101.8 [2]
Rabbit (4)	917	107.0	145.0	5.0 [3]	764	14.1	17.0	115.0
Dog (5)	922	109.0	142.0	3.7	765	21.5	32.4	82.1

B. Derived data pertaining to muscle

ANIMAL	$(H_2O)_E^{Cl}$	$(H_2O)_C$	Excess Na	$[Na]_C$	$[K]_C$
	gm/kg		m.eq./kg	m.eq./kg $(H_2O)_c$	
	(9)	(10)	(11)	(12)	(13)
Chicken, 3–4 day	514	313	18.5	58.6	140
Adult	70	671	5.7	8.5	147
Frog	140	660	11.4	17.3	125
Rat, 15 day	329	468			
Adult	144	629	1.6	2.6	154
Rabbit	129	635	0	0	180
Dog	193	572	6.6	11.5	142

[1] Concentration in extracellular fluid.

[2] Units per kilogram fat-free wet weight (fat content usually 1–2%).

[3] An assumed value.

References: (1) Fenn, '36b; (2) Hines and Knowlton, '39; (3) Miller and Darrow, '40; (4) Manery and Hastings, '39; (5) Hastings and Eichelberger, '37.

respectively. Comparison with the figures in table 3 A indicates that this treatment of chicks did not decrease the variability of the data, and by the application of Fisher's "F" test (Goulden, '39, p. 38) this has been shown statistically to be the case. The variability is undoubtedly due to the differences in growth rates common to young animals. The average values obtained from the two sets of data suggest that some dehydration occurs, particularly in muscle, in the group not given water. Fisher's "T" value for muscle water was 3.2 (Goulden, '39, p. 120), which showed that the observed difference between averages was significant. For this reason, in all subsequent experiments chicks were placed in a second incubator, immediately after hatching, and were given water *ad libitum.*

For the data given in table 3, 22 groups totalling 84 three day old chicks were used. The sera and breast muscles were analyzed for sodium, potassium, chloride and water. The average chloride concentration in muscle of 68.2 m. eq. per kilogram is somewhat higher than that of 52.8 which was previously reported (Barlow et al., '47); the difference can be attributed to the fact that the chicks formerly described were a day or so older than the ones used in the present research. In the same table the analyses of young chick serum and muscle are compared with those of adult chickens and with data taken from the literature on the composition of skeletal muscle of other young and adult animals. The sera of the young chickens and the adults have approximately the same composition, but striking differences appear in the composition of young and adult muscle. The muscle of the young chick had 9% more water than that of the adult, 7 times the chloride concentration, about 6 times the sodium, and one-half the potassium of the adult muscle. The muscle of young rats likewise has appreciably more chloride and water than the adult rat muscle. All of the other species listed in the table have, like the adult chicken, a low muscle chloride. Similarly, high potassium and low sodium concentrations seem to be characteristic of muscles of fully grown organisms regardless of species, but are not found in young chick muscles.

Because of the two types of evidence for the extracellular position of chloride which have already been presented we felt justified in using the muscle chloride to calculate the volume of extracellular water; having done this we estimated intracellular water volume and the concentrations of electrolytes inside cells (table 3 B). The high chloride content of the young muscle caused the calculated extracellular water to amount to 514 ml/kg, with a range of 407 to 609. Thus 50% of the muscle is extracellular space, a much larger proportion than the 7% found in adult chick muscle. It has already been shown (Barlow et al., '47) that most of the decrease to the adult value occurs in the first month after hatching. Of all the animals listed in the table only young rats have such a high proportion of muscle extracellular water.

The high chloride content of young organisms of many species has been recorded before (Irving and Manery, '36), although it was not at that time related to the extracellular space. It is of some significance, therefore, that more recently the extracellular water has likewise been found to be larger in infants and children than in adults. The volumes of distribution of injected thiocyanate (Robinow and Hamilton, '40; Morse et al., '47; Doxiadis and Gairdner, '48), Na^{24} (Flexner et al., '47) and thiocyanate and Na^{24} (Fellers et al., '49) have been measured in order to secure this information. Since muscle, which we have shown to possess a large histological extracellular space, comprises a large proportion of the total body weight, much of the decrease in extracellular water during development must have occurred in the muscular tissue. It is reasonable to conclude that muscular development in many species is characterized by the displacement of extracellular fluid by tissue cells.

Subtracting the value obtained for the extracellular water from the total water of the muscle gives an estimate of 313 ml of cell water per kilogram of muscle for the young chick and 671 ml of cell water per kilogram of muscle for the adult chicken. When the sodium and potassium not accounted for in the extracellular fluid are located inside the cell, we obtain the values given in columns 12 and 13 of table 3 B for the concentrations of sodium and potassium in the water of the cells. All the muscles show a high concentration of potassium in the cell water. In the chicken muscle, the cell potassium is approximately the same in the young and adult muscle (140 and 147 m. eq./liter of cell water respectively). However, the calculated sodium concentration of the muscle cells of the young chicken (58.6 m. eq./liter of cell water) is apparently much higher than the sodium concentration in the muscle cells of the adult (8.5 m. eq./liter of cell water). Table 3 B also shows wide species differences in the concentration of the so-called intracellular sodium from 17.3 m. eq./liter in the cell water of the frog to 0 in the rabbit. The absolute amount of this sodium per kilogram of muscle which is not accounted for in the extracellular fluid is labelled ''excess Na'' and is shown in column 11 of table 3 B. Since there is much more cell water (table 3 B, col. 10) per kilogram of adult than of young muscle and since, according to our calculations, we have tentatively allotted the ''excess'' sodium to the cells, the apparent intracellular sodium concentration (col. 12) becomes by calculation much greater in the baby chick muscle than in hen muscle. We do not know where this extra sodium is located but the disparity between the sodium concentration and that of chloride is assured (see cols. 6 and 7). If it were not inside cells but bound to some extracellular solid the development of muscle could be simply visualized as a displacement of the extracellular phase by a cellular phase which had a constant composition throughout the growing period.

SUMMARY

1. The serum and breast muscle of chicks three to four days old were analyzed for sodium, potassium, chloride and water. Analyses of 22 groups totalling 84 chicks indicated that the serum contained 93.7% water, 129.8 ± 7.4 m. eq. of chloride,

158.6 ± 7.6 m. eq. of sodium, and 7.0 ± 1.2 m. eq. of potassium per liter of water. The muscle contained 82.7% ± 1.6 water, the concentration of chloride was 68.2 ± 7.3 m. eq., of sodium 96.6 ± 7.2 m. eq., of potassium 45.5 ± 4.9 m. eq. per kilogram of fresh tissue. A comparison of these values with those of adult chickens showed that the sera of the two age groups have approximately the same composition; the muscle of the young chick, however, has about 9% more water, 7 times the chloride concentration, about 6 times the sodium and one-half the potassium concentration of the adult muscle. During development sodium and chloride decreased while potassium increased in concentration. These observations suggested that extracellular fluid was displaced by cells as the muscle developed.

2. Evidence that the muscle chloride was largely confined to the extracellular phase which comprised about 50% of the volume of the muscle was obtained in two ways.

(a) The "chemical" cell volume (based on the chloride concentration of muscle and serum) differed from the "morphological" cell volume (based on planimeter measurements of projected histological sections) by an average of + 1.3% of the wet weight of the muscle in 9 pairs of contralateral muscles.

(b) When breast muscles were soaked in modified Ringer's solutions at 37°C. for 40 minutes a linear relation was obtained between the chloride concentration of the muscle and that of the medium.

3. The chick breast muscle gained weight when soaked in hypo-, iso- or hypertonic Ringer-Dale solutions. Muscles in hypotonic media had smaller extracellular fluid volumes, $(H_2O)_E^{Cl}$, than their contralateral controls in isotonic solutions. Conversely those in hypertonic solutions had more extracellular fluid than their controls. These changes can be interpreted to mean that the muscle cells swell at the expense of the extracellular fluid in hypotonic media and shrink, their volume being replaced by extracellular fluid, in hypertonic media.

4. Using chloride to calculate the volume of extracellular water the following histochemical picture appears. Breast muscle of the young chick contains 514 ml extracellular water and 313 ml of cell water per kilogram. The calculated intracellular potassium concentration is about 140 m. eq. per liter of cell water and does not differ appreciably from that of muscle cells of the adult. The muscle of the young individual contains more excess sodium than that of the adult; its location is discussed.

ACKNOWLEDGMENTS

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GROWTH PROMOTING AGENTS IN CELL FRACTIONS OF CHICK EMBRYOS[1]

R. J. KUTSKY[2] AND MORGAN HARRIS
Department of Zoology, University of California, Berkeley

INTRODUCTION

The use of tissue extracts to stimulate the growth of animal cells *in vitro* was introduced by Carrel in 1913 and has become a classic feature of conventional culture methods. Extracts are commonly prepared from chicken or beef embryos (Parker, '50) although growth effects have also been reported using preparations obtained from various adult organs including heart and brain (Hoffman, Tenenbaum and Doljanski, '39; Davidson and Waymouth, '45; Hoffman, Dingwall and Andrus, '52). The identity of the specific growth factors in embryo or other tissue extracts is not as yet clear, although the view has been expressed (Fischer, '46) that these include non-dialyzable agents as well as smaller accessory factors.

The technique of ultracentrifugation has been used by several workers to investigate growth-promoting agents in tissue extracts. Tennant, Liebow and Stern ('41) as well as Fischer and Astrup ('43) subjected chick embryo extracts to ultracentrifugation and found that both pellet and supernatant stimulated growth of chick fibroblasts. These results coincide with the work of Barski, Wielgosz and Lepine ('51) who centrifuged saline extracts of chick embryos at 173,000 g for 4 hours and assayed the final supernatant in culture. The supernatant was less active than the original extract although a con-

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[2] AEC Predoctoral Fellow in the Biological Sciences, 1951–53.

siderable fraction of the activity was not sedimented under the conditions used. The pellet was not tested directly. Hoffman, Dingwall and Andrus ('52) prepared saline extracts from chick embryos and adult sheep heart, obtaining a supernatant and pellet in each case by ultracentrifugation at 30,000 r.p.m. for one to three hours in an air-driven centrifuge. Assays with chick fibroblasts indicated some activity in the sedimentable fraction although the major growth-promoting effect remained with the supernatant phase. Recently Wolken ('52) has fractionated chick embryo extract by centrifuging at 33,450 r.p.m. in a Spinco Ultracentrifuge for periods up to 12 hours. Although this study was concerned in the main with chemical and physical characterization of the fractions, a few assays were made with cultures of chick heart. No significant differences in surface area at 48 hours were apparent in cultures supplemented with the high speed pellet and supernatant respectively.

It appears desirable to refine the results of these ultracentrifugal studies to correspond with specific cell fractions of the chick embryo. Separation of cell components by differential centrifugation was introduced by Bensley and Hoerr ('34) and by Claude ('38), using saline extracts. Subsequently sucrose solutions were found by Hogeboom, Schneider and Pallade ('48) to give better preservation of isolated intracellular components, and corresponding methods were devised for the separation of nuclei, mitochondria, microsomes (submicroscopic particles) and soluble material (final supernatant). The expanding volume of work on cell fractions has been reviewed recently by Schneider and Hogeboom ('51).

The present paper deals with the preparation of specific cell fractions from 12–13 day chick embryos and individual assay of these materials in tissue culture for growth-promoting activity. Isolation of cell fractions was carried out in hypertonic sucrose solutions using modifications of the methods originally devised by Hogeboom, Schneider and Pallade ('48) for liver cells. The appropriate procedures and experimental results will be outlined in the following sections.

Isolation of cell fractions

Nuclei. Fresh 12–13 day chick embryos were removed aseptically from their membranes, eyes and legs excised, and blotted briefly on sterile filter paper. Each embryo was then placed in a glass homogenizer tube, 12.0 ml sterile 0.88 M-sucrose solution (pH 7) added and the mixture ground with a motor-driven pestle. The homogenates were pooled and strained through a double thickness of gauze followed by a single thickness of "Nu-Fab"[3] in order to remove larger debris. The strained homogenate was centrifuged at 600 g (1980 r.p.m.)[4] for 10 minutes using an International Model SB Type 1 centrifuge. To retard breakdown of cell particulates the medium was pre-chilled and maintained at 8°C. or less in this and subsequent operations. The initial pellet obtained contained cellular debris, red blood cells, and nuclei. The latter could be demonstrated microscopically in smears of this material by staining with pyronin methyl green. After resuspending the initial sediment three times in the original volume of sucrose solution and centrifuging as before, the nuclear fraction together with some erythrocytes became recognizable as a definite layer at the base of the centrifuge tubes. This layer was then removed for assay and just before adding to the culture medium was washed to remove the sucrose by suspending and centrifuging briefly in Gey's solution.

Mitochondria. The original nuclear supernatant was centrifuged twice at 600 g (1980 r.p.m.) to remove residual nuclei and debris. Following preliminary clarification as described above the supernatant was centrifuged at 24,000 g (13,700 r.p.m.) for 20 minutes. In this and in the following steps a Spinco Model L refrigerated preparative ultracentrifuge with angle head was used. The plastic centrifuge tubes required for high speed separations were sterilized by autoclaving using internal glass supports to prevent collapse. In operation each tube was sealed with a sterile metal cap. The yellow opaque pellet obtained represented the mitochondrial fraction as judged by the characteristic color and appearance and by microscopic observation of smears stained with Janus green. The pellet was then resuspended in a volume of fresh sucrose solution equivalent to the original supernatant and centrifuged as before. The final pellet was removed for assay and just before adding to the culture medium was resuspended and centrifuged in Gey's solution to remove sucrose.

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[3] Essential Products, Inc., Chicago, Illinois.

[4] Centrifugal forces specified in the present experiments are expressed as average values at center of tubes.

Microsomes. A fraction made up of submicroscopic particles (microsomes) was obtained by further processing of the supernatant from the mitochondrial separation. This supernatant was centrifuged for one hour at 105,400 × g (40,000 r.p.m.) resulting in the formation of a reddish, transparent pellet. This pellet was resuspended in the same volume of sucrose solution and again sedimented in the ultracentrifuge. Sucrose was likewise removed from this fraction just prior to testing by suspension in Gey's solution followed by ultracentrifugation.

Final supernatant. After separation of the various particulates the remaining fluid fraction (final supernatant) was preserved for assay of soluble factors not sedimented by the above procedures. This fraction was dialyzed against sterile Gey's solution to remove sucrose along with other dialyzable materials. Dialysis was carried out on a Boerner shaker in the cold room using three-quarter inch dialysis tubing (Visking Corp. N. Y.) immersed in a 2 l Erlenmeyer flask filled with Gey's solution. The dialysis units were sterilized before use by autoclaving. Two changes of dialyzing fluid were made over a period of 24 hours. The dialyzed supernatant was removed by pipette and stored in rubber stoppered tubes prior to use.

Assay procedures

All cultures were made in Carrel flasks using fresh explants from 12–15 day chick heart. The basal unsupplemented medium included a solid phase of chicken plasma and supernatant of dilute serum (40% horse serum, 60% Gey's saline). The following were incorporated into the saline fraction so as to give the corresponding levels in the completed medium: 100 units/ml sodium penicillin G (Merck), 50 units/ml dihydrostreptomycin (Pfizer) and 0.002% phenol red. The concentration of whole saline embryo extract (EE_{50}) in the medium was restricted to a minimum required for the initial clotting process (approximately 0.01 ml EE_{50} per flask). Supplements to be tested were added in an appropriate volume of Gey's solution which ordinarily constituted one-fourth of the saline fraction. The relative concentrations of specific cell fractions were expressed in terms of corresponding amounts of starting material (embryo equivalents) per series of 6 flasks. The cultures were incubated at 38°C. and supernatants replaced biweekly without washing the plasma clot. Outgrowths were examined microscopically at regular intervals and areal tracings made with a projectoscope. Areal increases, between zero and 10 days, were determined with a planimeter and the results treated statistically. Reference may be made to a preceding paper (Harris, '52a) for a more detailed description of assay procedures.

Supplementation with specific cell fractions

Controls. Outgrowths in unsupplemented media provided a basal level of reference for the various experimental series. In such cultures the increase in surface area (table 1) was minimal over the 10-day assay period. Although occasional explants exhibited thin, widely spreading mesothelial sheets, the usual pattern was a characteristic compact outgrowth zone. The cells were massed closely, became granulated and refractile after a few days, and did not show radial orientation with respect to the explant.

Nuclei. Washed nuclear fractions were added to assay cultures at levels of 5, 7, and 15 embryo equivalents per series of 6 cultures. None of these concentrations produced significant changes in surface area (table 1) and the cell picture microscopically was identical with that of control cultures. These results suggest that the nuclear fraction is inert but not inhibiting in culture.

Mitochondria. Washed mitochondrial fractions exhibited a clear-cut growth-stimulating effect when tested alone in assay cultures. This could be verified by measurements of surface area (table 1) as well as by correlated microscopic changes. Supplemented cultures showed a predictable shift from controls in cell picture and colony pattern. The cells in the outgrowth zone were visibly more numerous, did not mass closely

TABLE 1

Effect of cell fractions from chick embryos on areal spread of chick heart fibroblasts[1]

CELL FRACTION ADDED	CONCENTRATION LEVEL, EMBRYO EQUIVALENTS [2]	AREAL INCREASE [3] AT 10 DAYS	DIFFERENCE FROM UNSUPPLEMENTED PAIRED CONTROLS	SIGNIFICANCE OF DIFFERENCE, P
		mm^2		
None (controls)	...	13.2 ± 1.7	...	..
	...	16.0 ± 2.7	...	..
	...	16.0 ± 5.7	...	..
	...	10.8 ± 1.7	...	..
	...	18.0 ± 2.3	...	..
Nuclei	5	14.5 ± 1.0	— 1.5 ± 2.9	> 0.5
	7	16.7 ± 0.9	— 0.5 ± 1.9	> 0.5
	15	18.1 ± 1.8	0.9 ± 2.5	> 0.5
Mitochondria	3	25.1 ± 1.6	14.3 ± 2.4	< 0.001
	5	37.8 ± 3.7	21.8 ± 4.6	< 0.001
	5	33.7 ± 5.3	22.9 ± 5.6	< 0.01
	8	54.9 ± 0.9	44.1 ± 1.9	< 0.001
	8	51.4 ± 1.3	35.4 ± 5.8	< 0.001
	8	40.7 ± 1.0	22.7 ± 3.0	< 0.001
	14	21.9 ± 0.6	8.7 ± 1.8	< 0.01
Microsomes	4	55.7 ± 1.9	43.1 ± 7.5	< 0.001
	8	56.0 ± 2.4	44.4 ± 7.7	< 0.001
	13	29.9 ± 2.3	16.7 ± 2.9	< 0.001
Dialyzed final supernatant	0.5	55.7 ± 4.0	42.5 ± 4.4	< 0.001
	0.3	59.6 ± 3.2	50.5 ± 2.9	< 0.001

[1] *Medium:* Chicken plasma clot + 40% horse serum, 60% Gey's solution.

[2] Total amount added per series of six cultures.

[3] Mean value and standard error (Snedecor, '46). Six cultures per series.

together, and showed a more regular, radial alignment, particularly at the periphery of the outgrowth zone. After a few days the outgrowths became refractile and vacuolated but continued to expand. In general appearance these outgrowths closely resembled those previously obtained by supplementing serum cultures with dialyzed whole embryo extract (Harris, '52a).

The effects of supplementation with mitochondrial suspensions were most clearly evident at dosage levels corresponding to 8 embryo equivalents per series, falling off below this point (table 1). High concentrations of mitochondria produced lesser increases in surface area with the cells increasingly refractile and heavily loaded with vacuoles. These inclusions may reflect the relative abundance of lipids in the cytoplasmic particulates of chick embryos as reported by Claude ('40).

Microsomes. The addition of washed suspensions of microsomes produced large increases in surface area (table 1) and cellular changes similar to those described for cultures supplemented with mitochondria. No significant differences were observed in comparisons of cultures supplemented with the two particulates respectively. As with mitochondrial suspensions, high levels of microsomes were inhibiting.

Soluble cell fraction (dialyzed final supernatant). The final supernatant retained a conspicuous level of activity after separation of the particulate fractions and dialysis to remove sucrose and other components of low molecular weight. Even relatively minor additions of this fraction produced substantial increases in surface area (table 1) and altered growth pattern. In general these changes were similar to these produced by suspensions of mitochondria or microsomes but the outgrowths showed fewer inclusions, were less refractile, and the cells more regular in arrangement. It is apparent that while suspensions of cytoplasmic particulates exhibit growth-promoting effects, the largest part of the activity of whole embryo extract resides in the soluble cell fraction.

Solubilization experiments with mitochondria

Additional studies were carried out to test for the release of growth-promoting agents in soluble form from washed suspensions of mitochondria. These were obtained as pellets by the procedure outlined above and were extracted by the following alternative treatments:

1. *Treatment with distilled water.* Eight embryo aliquots of mitochondria were washed briefly in double distilled water and then resuspended in 12.0 ml distilled water for 24 hours at 0°C. The suspension was then centrifuged one hour at 105,400 g (40,000 r.p.m.) and the pellet discarded. The supernatant extract was made isotonic by appropriate addition of concentrated Gey's solution and assayed in culture. As a variant of this procedure suspensions of mitochondria in distilled water were frozen and thawed two or more times before the final centrifugation.
2. *Freezing and thawing in Gey's solution.* A second procedure involved resuspension of washed mitochondria (8 embryo equivalents) in 12.0 ml Gey's solution after which the preparation was frozen and thawed twice or more and finally centrifuged at 105,400 g for one hour (40,000 r.p.m.). The supernatant extract was used subsequently for assay.
3. *Extraction with 70% ethanol.* Mitochondrial pellets containing 8 embryo equivalents were washed in Gey's solution, resuspended in 25.0 ml of 70% ethanol and placed in the refrigerator at 0°C. for 24 hours. After centrifuging this suspension for one hour at 105,400 g (40,000 r.p.m.) the supernatant was evaporated over $CaCl_2$. The residue was reconstituted for assay purposes with Gey's solution.

Table 2 summarizes in part the results obtained from assays with these extracts. In general changes in surface area were paralleled by alterations in cell picture as described for intact suspensions of mitochondria. It appears that the active principle in mitochondrial fractions can be partially solubilized by treatment with either distilled water or Gey's solution although there was no clear indication that extraction was aided by freezing and thawing. Extraction with 70% ethanol did not solubilize active materials and this result is particularly interesting since alcoholic homogenates of entire embryos are rich in dialyzable growth factors (Harris, '52b, '53).

These observations suggest the possibility that one or more growth factors present in the dialyzed soluble cell fraction may be identical with those of the cytoplasmic particulates. The significance of this point, however, remains to be assessed. It is possible that the activity associated with the mitochondria and microsomes stems from material adsorbed from the soluble cell fraction, rather than from factors intrinsic to these particulates as such. This view is consistent with the large aggregate surface area of the mitochondrial or microsomal fractions, and the considerable disparity in specific activity between the particulates and soluble cell fraction (table 1). On

TABLE 2

Effect of extracts obtained from washed mitochondrial suspensions on areal spread of chick heart fibroblasts[1]

EXTRACTION MEDIUM	ADDITIONAL TREATMENT	AREAL INCREASE[2] AT 10 DAYS IN CULTURES SUPPLEMENTED WITH FINAL CENTRIFUGED EXTRACT	DIFFERENCE FROM UNSUPPLEMENTED PAIRED CONTROLS	SIGNIFICANCE OF DIFFERENCE
		mm^2		
Distilled water	None	36.5 ± 4.9	18.5 ± 5.4	< 0.01
	Frozen and thawed once	49.4 ± 2.5	38.6 ± 3.0	< 0.001
	Frozen and thawed twice	30.0 ± 3.0	19.6 ± 3.2	< 0.001
Gey's solution	None	33.3 ± 2.1	22.9 ± 2.2	< 0.001
70% ethanol	Evaporated; reconstituted in Gey's solution	17.6 ± 2.8	— 0.4 ± 3.6	> 0.5

[1] *Medium:* Chicken plasma clot plus supernatant of 40% horse serum, 60% Gey's solution.

[2] Mean value and standard error (Snedecor, '46). Six cultures per series.

[3] Added at levels corresponding to eight embryo equivalent per series.

the other hand the possibility cannot be excluded that the growth factors in question may in fact originate from cytoplasmic particulates with progressive release from these centers in soluble form. While additional experiments will be required to explore this possibility, further investigation is warranted in the light of proposals by Brachet ('50) and others that cytoplasmic particulates may play an important role in protein synthesis, i.e. — growth.

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SUMMARY

1. Chick embryos of 12–13 days' incubation were homogenized in hypertonic sucrose solution and separated into cell fractions by differential centrifugation. Nuclear, mitochondrial, microsomal, and dialyzed soluble cell fractions were obtained.

2. The growth-promoting effect of each fraction was assayed in cultures of chick heart fibroblasts. Cell appearance, colonial pattern, and surface area were used as criteria.

3. The nuclear fraction was inert but not inhibiting. Washed mitochondrial or microsomal suspensions produced conspicuous increases in surface area accompanied by characteristic alterations in cell morphology and cultural pattern. Similar changes were obtained on supplementation with the dialyzed soluble cell fraction. The dialyzed soluble cell fraction was active in high dilution and at equivalent concentrations was more effective than suspensions of the cytoplasmic particulates.

4. The active principle in mitochondrial suspensions can be partially solubilized by treatment with distilled water or Gey's solution but is not extracted with 70% ethanol.

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THE EFFECT OF NITROGEN MUSTARDS ON CELL DIVISION IN CHILOMONAS PARAMECIUM AND SACCHAROMYCES CEREVISIAE [1, 2]

JOHN O. HUTCHENS AND BETTY PODOLSKY
Department of Physiology and the Toxicity Laboratory,
University of Chicago, Illinois

SEVEN FIGURES

INTRODUCTION

The nitrogen mustards (β-chloroethyl amines) and sulfur mustard [bis-(β-chloroethyl) sulfide] have come to be of interest in a variety of areas of biology. Their abilities to produce gene mutations (Auerbach et al., '47; Horowitz et al., '46; Stahmann and Stauffer, '47; Miller and McElroy, '48), block cell division (Friedenwald et al., '47; Hutchens and Podolsky, '48), inhibit embryological development (Bodenstein and Kondritzer, '48) and inhibit a variety of enzyme systems (Gilman and Philips, '46 [3]; Barron et al., '48) are of perhaps greater current interest than their clinical usefulness in blood dyscrasias. In spite of extensive investigations during and since World War II, the exact details of their effects on individual cells and the mechanisms by which these effects are produced are poorly understood. Biological effects of the nitrogen and sulfur mustards have been attributed to the alkylating ability of the ethylene immonium or ethylene sulfonium cations respectively (Gilman

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[3] This paper, a review, refers to research by various workers, much of which is to be found in detail only in classified literature which was available to us but cannot be specifically cited.

and Philips, '46). Beyond this, however, much doubt exists as to the nature of the systems attacked.

Previous investigations of the effects of mustards on growth and metabolism and the mechanisms of their production have involved a wide variety of experimental material including yeast (Kinsey and Grant, '47), amphibian embryos (Bodenstein, '47), and excised mammalian tissues (Barron et al., '48; Cori et al., '45). Each of these offers advantages but suffers severe limitations for studies of growth and cell division. For example, "surviving" mammalian tissues are, in reality, dying in the course of an experiment and at best have very limited capacity for anabolic activity. Yeast overcomes this difficulty but frequently fails to divide completely (budding) and offers no karyokinetic picture resembling that seen in animal cells. Embryos consist of a heterogeneous cell population of varying susceptibility to mustards.

Our previous investigations (Hutchens et al., '48) of the metabolism of *Chilomonas paramecium* encouraged us to believe that experiments using this organism would yield results easier of interpretation than had been possible with other types of material. *Chilomonas* grows readily in a simple medium (Hutchens, '48) the components of which would not be expected to react rapidly with mustards. The organism is motile and presents a well-defined cytological picture including a mitotic process closely resembling that seen in most animal cells. The normal rate of cell division, the rates at which various protoplasmic constituents (starch, fat, protein) are synthesized, and the rates at which metabolites are utilized are well established for *Chilomonas*.

We therefore undertook an investigation of the effects of tris(β-chloroethyl) amine (HN-3) and bis(β-chloroethyl) methylamine (HN-2) on rates of cell division, respiration, acetate and ammonia utilization, and of carbohydrate and protein synthesis and breakdown by *Chilomonas*. In addition, baker's yeast has been used for comparative studies on cell division, fermentation, and protein and nucleic acid synthesis. The present paper deals with the effects of HN-2 and HN-3 on cell division. Their effects on metabolism are reported in an accompanying paper.

MATERIAL AND METHODS

The chilomonads used were of the same strain used for our investigations of the kinetics and energetics of carbon and nitrogen metabolism. Methods of culturing, handling, and counting the chilomonads have been described (Hutchens, '48; Hutchens et al., '48).

The yeast used was a pure clone culture isolated from commercial (Fleischmann's) baker's yeast. The yeast was grown in a medium containing 2% sucrose, 0.2% yeast extract (Difco), 8.6 mM NH_4Cl, and 8.6 mM KH_2PO_4 in redistilled water at pH 6.0. During growth experiments the yeasts were kept at 37°C. by immersion of the tubes containing the suspensions in a thermostated water-bath. Air, saturated with water vapor at 37°C., was bubbled through the suspensions. (In spite of the aerobic conditions, as is shown in the accompanying paper, these yeast cells fermented sucrose but did not oxidize the breakdown products.)

The number of yeast cells present was estimated by measuring the optical densities of the suspensions at 600 mμ using a Coleman Junior spectrophotometer. Optical densities generally were kept between 0.1 and 0.5 (19 mm round cuvettes) by diluting a suspension when it approached the upper level. This procedure, while adequate for control suspensions, leaves something to be desired when mustards are used, since budding and chain formation produce light-scattering entities without cell division. Perhaps even more important, the method does not distinguish dead from live cells. The technique, however, is essentially the same as that used by Kinsey and Grant ('47) and the results are directly comparable.

To establish the fraction of viable yeast cells (i.e., capable of producing colonies) present at any given time, suspensions were made containing 2% agar in the culture medium described. A drop of suspension was put on a warm slide and a coverslip added. The slides were incubated for 12–24 hours and 100 cells plus colonies counted differentially.

The HN-2 and HN-3 used were purified by distillation of the free bases and precipitation of the hydrochlorides by bubbling HCl gas through a solution of the free base in diethyl ether. The salt was always dissolved in distilled water immediately before use and amounts required to give the final desired concentration were added to the suspensions of organisms. Usually the volume of the amine hydrochloride solution added was 1% of the final volume of culture solution. Even when 4 or 5 serial dilutions were made, less than 5 minutes elapsed from original solution of the amine until addition of all of the solutions to the cultures. Considerable cyclization no doubt occurred in this time but hydrolysis must have been negligible (Golumbic et al., '46a, '46b). Unless otherwise stated the mustard was allowed to remain in the solution, i.e., the cells were not washed.

In certain experiments, chilomonads were washed after a one-hour exposure. This produces nearly the maximal effect obtainable with a given concentration. With yeast the effects probably were slightly mitigated when dilution occurred as early as two hours after exposure (see below).

EXPERIMENTAL RESULTS

The investigations reported here were conducted over a period of some three years. At the same time, studies of the effects of HN-2 and HN-3 on metabolism and synthesis were being conducted. No attempt has been made in the following account to present experiments in the chronological order in which they were performed. In particular, since HN-2 and HN-3 produce effects which are qualitatively indistinguishable and unless otherwise stated are equally effective at a given molar concentration, results with only one substance are presented in most cases.

Preliminary experiments with Chilomonas at 25°C.

We sought first to establish the range of concentrations of HN-2 and HN-3 within which effects on cell division were produced. A preliminary experiment demonstrated that 10^{-3} M HN-3 killed the exposed chilomonads in about one hour. At 10^{-4} and 10^{-5} M HN-3, cell division was inhibited as evidenced by cell counts made at 16 and 41 hours after exposure. Periodic inspection of these cells, both in the living condition and after staining with Lugol's solution, showed that, at 5–10 hours after exposure, they were larger than the controls and apparently contained more starch. HN-3 at 10^{-6} M did not reduce below the control level the number of cells produced at 16 or 41 hours.

A similar exploratory experiment with HN-2 yielded the following results: 10^{-2} M HN-2 killed the cells in about one hour. At 3.3×10^{-3} M, the cells were dead in about 8 hours; at 1.1×10^{-3} M much cytolysis was noted at 8 hours and all the cells were dead by 19 hours. At concentrations of 3.7×10^{-4} M, 1.2×10^{-4} M, and 4×10^{-5} M HN-2, cell division was inhibited, the cells becoming large and starch-filled as when exposed to HN-3.

Additional preliminary experiments disclosed that, in the range 10^{-6}–10^{-4} M, both HN-2 and HN-3 effected a block to cell division which was not relieved until some 10–12 hours after exposure at 25°C. We therefore undertook a detailed study of the effect of various concentrations of the mustards within this range on the time course of cell division.

The time course of cell division in Chilomonas following exposure to mustards at 25°C.

Figure 1 presents the results of two experiments conducted using HN-2 at various concentrations. The number of cells arising per original cell (n_t/n_o) is plotted as a function of the mean control division time. In these two experiments at 25°C., the control division time varied from 5.5–7.0 hours, fluctuating with the degree to which the cultures were disturbed by sampling and the magnitude of the population. None of the curves except that for 10^{-4} M HN-2 involves any cell deaths. (At least no "ghosts" or cell debris were observed at lower concentrations.) They therefore represent the true rates of cell division.[4] With 10^{-4} M HN-2, after cell division resumes at about two control division times, abnormal and cytolyzed cells were seen. The dip in this curve and the subsequent decreased slope represent the resultant of cell divisions and cell deaths.

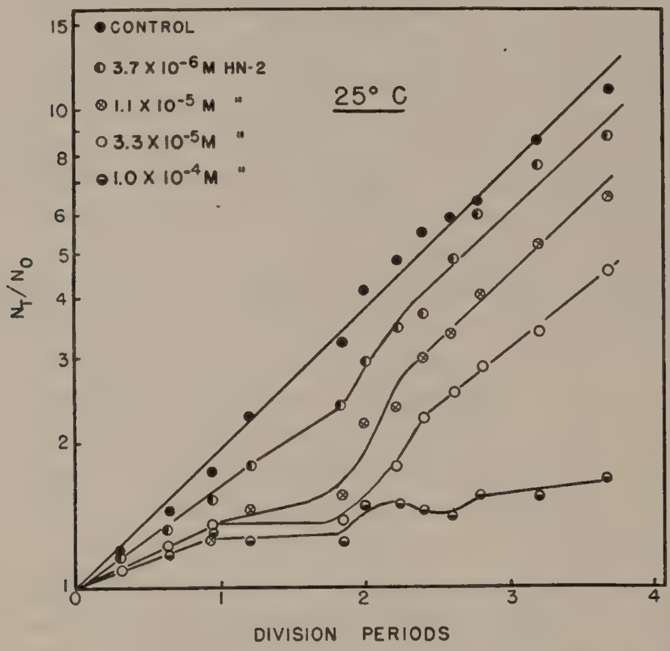

Fig. 1 Effect of various concentrations of HN-2 on cell division in *Chilomonas* at 25°C. One control division time equals ca. 6 hours.

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[4] Daniels, '52, using the giant amoeba, *Pelomyxa carolinensis*, in isolation cultures which permitted observation of individual deaths, obtained curves essentially like figure 1. Negligible cell death occurred.

These curves, which are almost exactly mimicked by HN-3 at the same concentrations, exhibit several features worthy of note:

(a) With the concentrations employed, cell division does not stop immediately. This might be interpreted in various ways. One obvious possibility is that time is required for the mustard to reach and react with cell constituents. However (see below), if cells are exposed briefly to mustards, then washed, the effects on cell division are essentially the same as those noted in figure 1. Such experiments argue in favor of immediate damage to the cells. However, one cannot exclude the possibility that the mustards are quickly taken up or adsorbed by the cells to react later with critical constituents.

A second possibility is that the cells are immediately affected but the damage does not become manifest until some particular karyokinetic stage is reached and that cells beyond this stage can complete a division. This possibility cannot be entirely excluded, but if such a stage exists, it is cytologically indistinguishable from the typical interphase. At the time of exposure more than 90% of the cells are in interphase and the cells in which division is blocked appear also to be in interphase. Another possible explanation, and one which has appealed to us as a working hypothesis, is that a system in the cells is immediately affected but that a product of this system is present in some of the cells in sufficient concentration or amount to permit a division to occur.

(b) After a period of decreased rate of cell division, the rate of division of mustard exposed cells briefly exceeds and then returns to the control rate. Various possible explanations presented themselves:

1. Division is resumed when the mustard in the medium is exhausted. Experiments are presented below which demonstrate that an inhibitory material is present in the medium when cell division resumes.

2. A cell constituent is destroyed by the mustard and division is resumed only after it has been replaced.

Destruction of a cell constituent seems inconsistent with the graded dose-effect relations apparent from figure 1. If destruction implies irreversible combination (e.g., alkylation) or reaction with the mustard, it should proceed until one or the other of the reactants is exhausted. Since unreacted mustard is present for several hours or can be added (see below) at doses which produce less than the maximal effect, this hypothesis seems untenable. Furthermore, the presence of the unreacted mustard would require that the destroyed constituent be replaced with a non-reactive substitute which seems unlikely.

3. The mustard or a derivative (e.g., the cyclic onium cation) reacts reversibly with a cell constituent, establishing an equilibrium. Association with the mustard effectively removes the constituent (enzyme, metabolite, structural molecule) from participation in the cell's economy, and cell division is blocked. Continuing synthesis at steps preceding the affected one generates material which alters the equilibrium and permits division to proceed.

For example, if the affected constituent were an enzyme, the mustard might compete with the normal substrate for position on the enzyme, thus slowing the rate of the metabolic step. Continuing generation of the normal substrate by preceding steps could conceivably relieve the inhibition simply on a mass action basis. In addition, synthesis of more of the enzyme (and in the case of reversible reactions, removal of the product of the step) could also contribute to relief of the inhibition. If the constituent which reacts reversibly with the mustard be non-enzymatic, then relief of the inhibition would have to be achieved simply by generation of more of the constituent. In this case, without any change in reaction constants, more total material would mean more material free from combination with the mustard and available for normal metabolic reactions.

At present we favor the hypothesis that mustard combines reversibly with an enzyme. This is also consistent with the temporary acquisition of resistance to the division-blocking action of the mustards by *Chilomonas.* However, this hypothesis cannot be accepted without reservation until the failure of washing the cells to relieve the inhibition has been explained *(vide infra).*

(c) Increasing doses of mustards exert increasingly greater effects. Such a family of dose-effect curves is expected in one of two types of situation:

1. The added agent reacts with a cell constituent or constituents until the agent is exhausted. Within a certain dose range the effect exerted will then be proportional to the amount of material added. Two facts argue against this being the explanation, although it is the one consistent with the hypothesis that the mustards act as alkylating agents. One fact is that the effect of a given concentration of mustard is independent of the concentration of cells over a wide range. The other fact, mentioned above, is that, up to 10 hours after addition of the mustard, it is present in the medium in active form.

2. As proposed above (b, 3), an equilibrium is established between a cell constituent and the inhibitor.

This explanation is consistent with the presence of excess unreacted mustard in the solution and with the ultimately achieved release of the cell division block.

The time course of cell division in yeasts exposed to HN-2

Figure 1 which represents the time course of cell division in chilomonads exposed to HN-2 or HN-3 bears a superficial resemblance to curves presented by Kinsey and Grant ('47) describing the time course of turbidity change in yeast suspensions exposed to bis(β-chloroethyl)sulfide. Certain differences between the curves relating to *Chilomonas* and those for yeast were apparent:

1. In Kinsey and Grant's experiments the yeast divided three or 4 times before there was any marked decrease in the apparent rate of cell division, as compared with less than one division obtained with *Chilomonas.*

2. Once division had slowed, it virtually ceased for several cell generation times.

We therefore decided to expose yeast to HN-2 to ascertain whether the differences were to be found when this substance was used. Figure 2 presents the results of an experiment in which yeast was exposed to various concentrations of HN-2. The curves are very similar to those obtained by Kinsey and Grant.

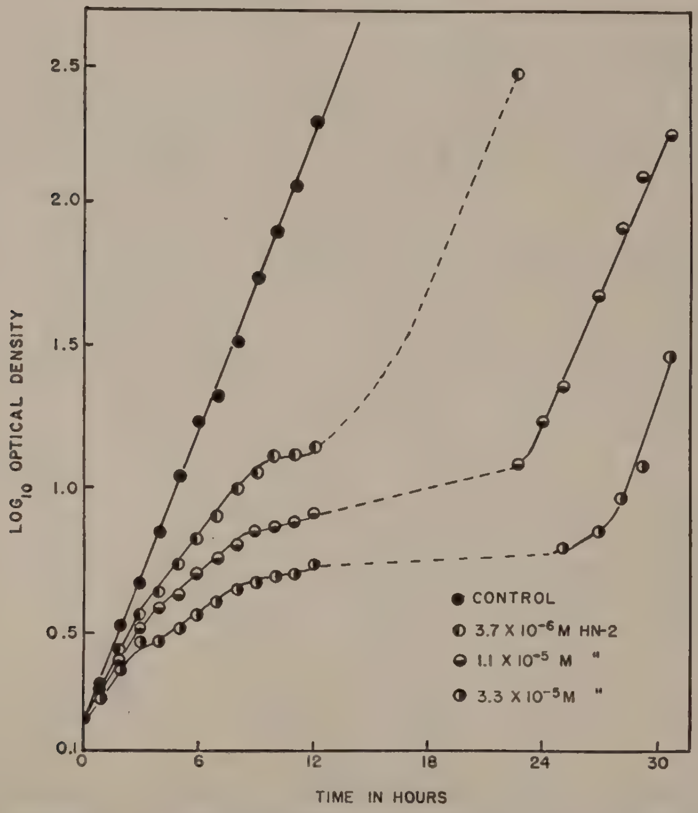

Fig. 2 Effect of various concentrations of HN-2 on optical densities of yeast cultures at 37°C.

One fact is striking. Whereas Kinsey and Grant had to employ concentrations of sulfur mustard of the order of

10^{-3} M, with HN-2 similar effects are obtained at 10^{-6}–10^{-4} M. This may be attributable to the short half-life of sulfur mustard as compared to HN-2 or to differing inherent potencies of the two compounds.

The question then arose as to the events responsible for the turbidity changes noted in figure 2. Optical densities give no indication of the fraction of viable cells present nor of the true course of cell division. There was reason to suspect from Kinsey and Grant's report that many of the yeast cells were not viable. We therefore prepared slides as described under *Methods* to ascertain the fraction of viable cells.

The results of the viability studies can be summarized briefly. Using 1.1 and 3.3×10^{-5} M HN-2 it was found that cells taken within one hour after exposure performed at least three or 4 divisions. Many of the colonies then ceased development or continued only at an attenuated rate. Samples taken at the time turbidity became constant, or was increasing only slowly, showed only about 10% of the cells exposed to 1.1×10^{-5} M HN-2 capable of developing colonies. With 3.3×10^{-5} M HN-2 the viable cells constituted only about 2% of the total. When the rate of turbidity increase was again near normal, a large fraction (70–90%) of the cells again yielded colonies.

It therefore appears that the resemblance of figures 1 and 2 is merely superficial. With *Chilomonas* we are dealing with a reversible block to the division of each cell. With the yeast the time course of turbidity increase reflects the death of a large fraction of the cells after three or 4 divisions followed by the slow development of a few survivors, until they replenish the culture with viable cells.

The effect of washing cells at various times after exposure to HN-2

As noted above, both chilomonads and yeast exposed to HN-2 or HN-3 continue to divide for some hours. A similar delay in onset of effect of mustards has been noted for corneal cells by Friedenwald et al. ('47). Delay in manifestation of toxic effects of both sulfur and nitrogen mustards in man (skin erythema, vesication, and systemic intoxication) is well known. We asked whether the delay in our experiments was to be attributed to slow reaction of the mustard with the cells or to some other cause. To answer this, cells were washed to remove the mustard from the ambient solution at various times following exposure. Both with yeast and chilomonads, the cells were packed once by centrifugation, the supernatant solution decanted, and fresh culture solution added to resuspend the cells. This reduced the HN-2 concentration to 1% or less of the original (a concentration less than the minimally effective one, cf. fig. 1).

The results of these experiments are presented graphically in figures 3 and 4. It is evident from figures 3 and 4 that washing at or before two hours following exposure to HN-2 protects yeast only partially and *Chilomonas* to a negligible extent. With *Chilomonas* a one-hour exposure produces virtually the full effect obtainable with the specified concentration even though the effect is not fully apparent until some hours later. With yeast the effect is greater the longer the exposure up to two hours, but here, too, the effect to be obtained is not apparent until some hours after washing. Barring the possibility that the HN-2 is adsorbed on the cells or enters to be held there, slowly reacting in subsequent hours, the experiments seem to eliminate delay in attack on the cells as the explanation for delayed manifestation of damage.

We are unable to choose between various possible explanations of the effect of washing HN-2 exposed yeast at various times, e.g., slow penetration, slow reaction, partial reversibility of effects. The experiment with chilomonads, on the other hand, seems to indicate rapid and irreversible damage to the cells. We are unable to reconcile this with our other experiments. The graded dose-effect curves and the recovery of the ability to divide in the presence of active mustard seem to argue for a reversible combination of mustard with a cell constituent. Why, if such a reversible combination is formed, washing does not lead to rapid recovery of ability to divide

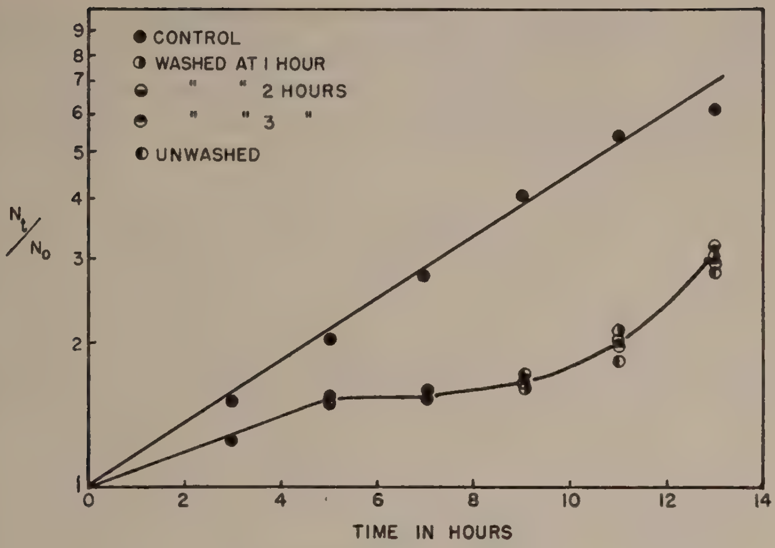

Fig. 3 Effect of washing chilomonads at various times after exposure to 3.3×10^{-5} M HN-2. T = 25°C.

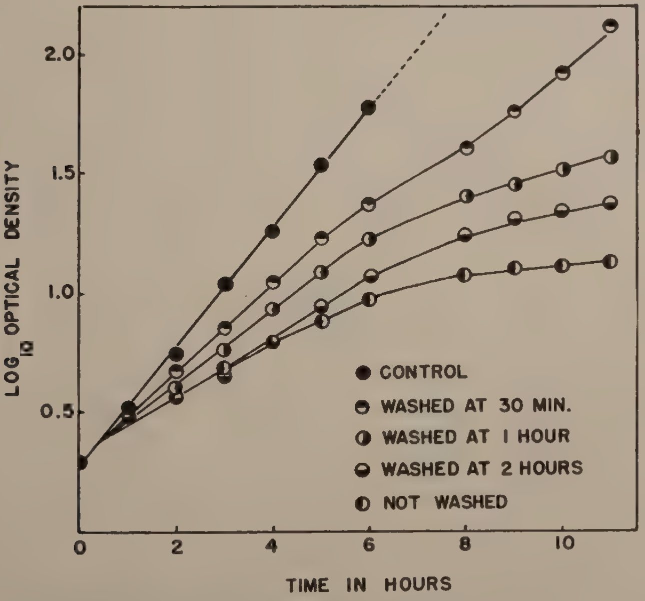

Fig. 4 Effect of washing yeast at various times after exposure to 1.1×10^{-5} M HN-2. T = 37°C.

is hard to explain. Therefore, this experiment involving washing of the HN-2 treated chilomonads remains as an embarrassing and potentially decisive contradiction to the hypotheses we have proposed to explain the bulk of our other experimental findings.

Duration of potency of HN-2 solutions

One of the most striking features of the curves presented in figure 1 is the abrupt resumption of cell division by chilomonads at 10–12 hours, i.e., ca. two division times, after exposure of the cells. Since we had not removed the mustards or their hydrolysis products, one possibility for explaining this phenomenon was that a period of 10–12 hours was required for hydrolysis of the chloroethylamine to non-toxic end products. This possibility was investigated by aging solutions of HN-2 for various periods before adding them to suspensions of chilomonads.

In the experiments involving aging of HN-2 solutions, a single concentration of HN-2, 6.6×10^{-5} M, in culture solution, was employed. The solutions were aged for times ranging from 15 minutes to 10 hours. Solutions were aged both in the presence and absence of chilomonads (ca. 5×10^4 cells/ml). At the end of the indicated periods the chilomonads, when present, were removed by centrifugation and aliquots of these solutions were added to an equal volume of a suspension of chilomonads, to give a nominal concentration of 3.3×10^{-5} M HN-2. Two controls were employed: (1) a suspension of unexposed chilomonads, and (2) chilomonads exposed to a freshly made 3.3×10^{-5} M HN-2 solution. A preliminary experiment in which solutions were aged 15, 30, 60, and 120 minutes showed that there was no detectable loss of potency within these time limits. The results obtained with longer aging are presented in table 1. There were no detectable differences in the solutions aged with and without cells and no distinction has been made between them in reporting results. As will be seen from table 1, some potency is lost in 5 or 10 hours, but material capable of inhibiting cell division is present for more than 15 hours. The observed effects strongly argued against the theory that resumption of cell division in the experiments presented in figure 1 is to be attributed to hydrolysis of mustards. The experiments presented in the next section demonstrated decisively that the cells recover the ability to divide even in the presence of active HN-2.

TABLE 1

Blocking of cell division by aged solutions of HN-2

(Original concentration, 3.3×10^{-5} M.) Temperature, 25°C.

HOURS POST EXPOSURE	SOLUTION AGED 0 hr.	5 hrs.	10 hrs.	15 hrs.
	% Control division			
0–5.5	37	55	62	80
5.5–8.8	22	38	48	58
8.8–11	20	58	91	..
11–12.8	60	56	..	..

Development and loss of resistance to cell division blocking action of HN-2

The experiments reported above in which it was found that active HN-2 was present in the culture medium at the time the chilomonads resumed division led us to suspect that they had developed a resistance to the cell division blocking action of HN-2. The possibility still remained that freshly added HN-2 would inhibit division further. We therefore performed the experiment presented graphically in figure 5.

A culture of cells was halved and one aliquot was exposed to 1.1×10^{-5} M HN-2. Twelve hours later the control cells had increased 5.5-fold, the exposed cells, 2.5-fold. This proportional development was that expected (cf. fig. 1). Each of the cultures was again halved and to one aliquot of each was added fresh HN-2 to give an additional 1.1×10^{-5} moles/l of HN-2. The previously unexposed cells were inhibited as expected. Those cells which had been in the HN-2 solution were unaffected by the HN-2. Some 12 hours later these cells were again exposed to a new dose of 1.1×10^{-5} M HN-2 without effect.

At the end of this experiment, a 1 ml sample of the triply exposed cells was added to 50 ml of fresh culture solution.

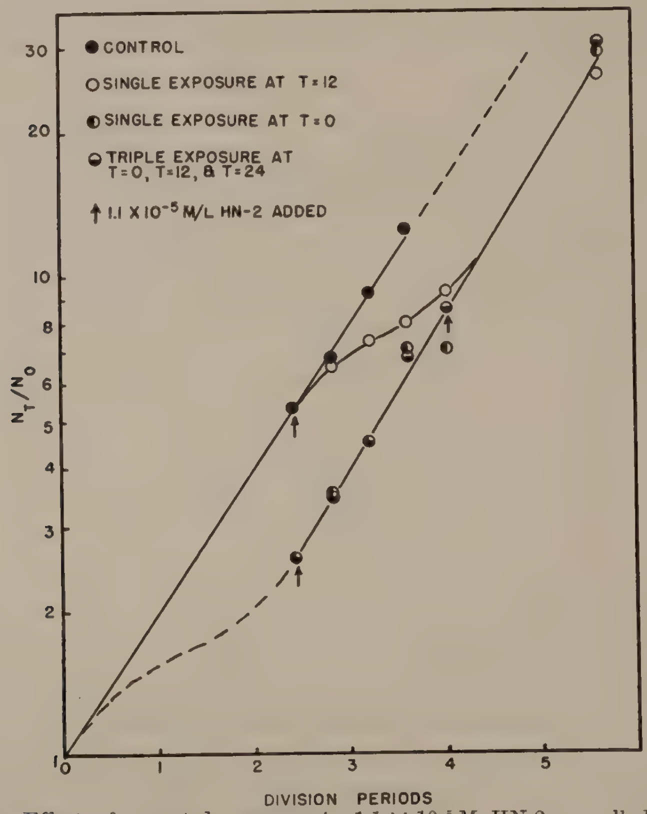

Fig. 5 Effect of repeated exposure to 1.1×10^{-5} M HN-2 on cell division in *Chilomonas.*

Nineteen hours later they had divided three times. They were again exposed to 1.1×10^{-5} M HN-2. At this time they were inhibited to the same extent as normal cells, i.e., the resistance had been lost.

It is to be emphasized that in this experiment at no time was any significant fraction of the cells killed. Selection is therefore not involved. Resistance to the action of HN-2 was uniformly acquired by all of the cells within one cell generation. This resistance was maintained as long as HN-2 was present. The resistance was lost on returning the cells to a mustard-free medium. No gene mutation seems likely.

To investigate the extent of the resistance to HN-2 acquired by the cells, we conducted the experiment presented in figure 6. A batch of cells in the logarithmic growth phase was divided into three parts. To one aliquot was added HN-2 · HCl sufficient to give 1.1×10^{-5} M HN-2. To another enough to

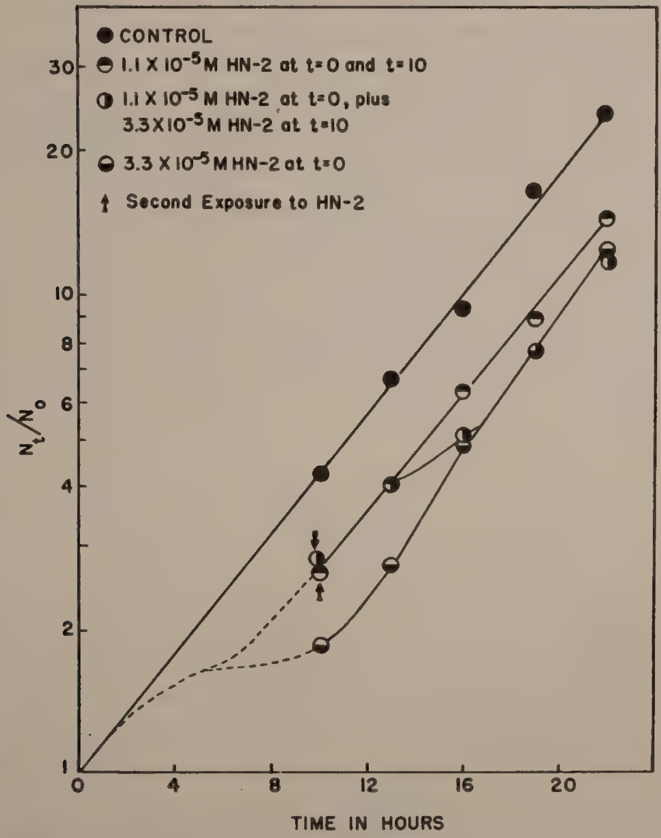

Fig. 6 Effect of repeated exposure to various concentrations of HN-2 on cell division in *Chilomonas*. (See text.)

give 3.3×10^{-5} M HN-2 was added. Ten hours later the culture originally exposed to 1.1×10^{-5} M HN-2 was halved. One half was exposed to a fresh dose of 1.1×10^{-5} M HN-2, the other to 3.3×10^{-5} M HN-2.

It will be seen from figure 6 that the cells exposed at $t = 0$ to 1.1×10^{-5} M HN-2 were, at 10 hours, resistant to a repetition of this dose as in the previous experiment. When exposed to three times the original concentration, namely 3.3×10^{-5} M HN-2, however, the cells suffered a further delay before resuming division. The remarkable fact is that this additional delay was just sufficient to make the curve representing this dosage schedule join the curve representing a dose of 3.3×10^{-5} M HN-2 at $t = 0$.

We consider these experiments to be strong supporting evidence for the hypothesis that escape from inhibition of cell division by HN-2 depends on accumulation of some material which competes with HN-2 for a critical cell constituent. At 10 hours enough material had accumulated to compete with 1.1×10^{-5} M HN-2, but not with three times as much. Having already accumulated some of the material, however, the time required to accumulate enough to compete with the higher dose was materially shortened. While there may be other tenable explanations for this phenomenon, none have occurred to us.

Effect of temperature on the time course of cell division in mustard-exposed cells

Further information bearing on the mechanism of the escape from the cell division blocking action of the mustards was obtained by varying the temperature at which the cells were exposed and incubated. *Chilomonas* grows and divides optimally at 25–27°C. (Smith, '40, and unpublished data of this laboratory). Of various methods available for slowing the metabolism of the cells, we felt that varying the temperature was technically most attractive and made the least threat of complicating the results.

The hypothesis which we wished to test was that the cells began to divide after metabolism had led to the synthesis of a given amount of some substance or substances. Choosing a temperature of 17°C. which Smith's curves indicated would approximately double the time required for cell duplication, we repeated the experiment presented in figure 1. The results are presented graphically in figure 7. Again the results are presented with cells arising from a single cell as a function of control division periods. At 17°C. one control division period equalled 12 hours.

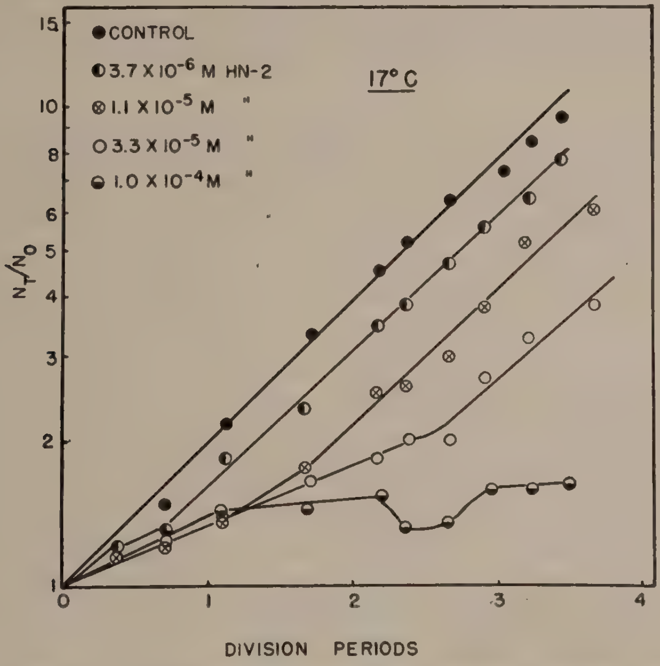

Fig. 7 Effect of various concentrations of HN-2 on cell division in *Chilomonas* at 17°C. One control division time equals ca. 12 hours.

Figure 7, while not an exact duplicate of figure 1, is very similar. Indeed, when the points obtained at a given concentration but at the different temperatures are fitted to a single curve, the deviations are slight. The chief difference lies in a less marked slowing of division at 17°C. after 30–50% of the exposed cells have divided. Two points of similarity of the curves deserve special notice:

1. At 1.1 and 3.3 × 10^{-5} M HN-2, cell division is markedly slowed only after 30–50% of the cells have divided. This is the same proportion noted at 25°C.
2. At these concentrations a normal rate of cell division is attained some 24 hours after exposure at 17°C. As at 25°C., this represents approximately two cell generation periods.

TABLE 2

Effect of exposure and incubation temperatures on division after exposure to HN-2

EXPOSURE TEMP.	INCUBATION TEMP.	HRS. TO FIRST DIV.	CONTROL DIV. PERIODS
25	25	10	2.36
25	17	27.5	1.77
17	17	21.5	1.67
17	25	9.2	1.73

The results, therefore, were not inconsistent with the idea that division resumed when metabolism had proceeded long enough to generate a given amount or concentration of a cell component. The possibility that the effects of temperature on the rates of cyclization, hydrolysis, and reaction with cell components of the HN-2 were responsible for the findings could not be ignored. We therefore conducted the experiment described below.

Two cultures of chilomonads were established, one growing at 17°C., the other at 25°C. The population of each was increasing exponentially, that at 25°C. at about twice the rate obtaining at 17°C. An aliquot of each was exposed to 1.1 × 10^{-5} M HN-2 for one hour at the appropriate temperature. They were then washed free of the HN-2 and aliquots of each were allowed to develop at both temperatures. Each batch of HN-2 exposed cells was compared to its individual control which had been grown at the same temperature, washed, and handled in an identical fashion. The results are presented in table 2.

It is apparent from table 2 that, whatever the exposure temperature, the cells recover more quickly at 25°C. than at 17°C. Centrifuging and temperature shock distorted the expected form of the growth curves, particularly in the case of the cells grown at 25°C. and transferred to 17°C. In spite of the complexity of the experiment, we take the results as essentially supporting the hypothesis that recovery of the ability to divide is correlated with the overall synthetic rates and not with the amount of damage done at the two temperatures.

Effect of various substances on the time course of cell division in Chilomonas following exposure to HN-2

In view of the results reported in the preceding sections, it seemed possible that by adding some material to the culture medium we might relieve the block to cell division caused by HN-2. Since HN-2 is believed to inhibit both protein and nucleic acid synthesis, we concentrated on precursors of these materials. Also, in the hope of finding an effective, even though unidentified, substance, we have tested yeast extract and autolysates of *Chilomonas.* Five-tenths per cent yeast extract or cysteine added to the medium before exposure exert a marked protective action, but this appears to be the result of chemical screening through reaction with HN-2.

Thus far the following substances added one hour after exposure to 1.1×10^{-5} M HN-2 have been tested with no beneficial effects noted: casein hydrolysate, 0.2%; yeast extract, 0.5%; water soluble substances obtained from chilomonads by freezing and thawing; the same, autodigested 12 hours at 25°C.; both of the previous with proteins removed by heat coagulation (the protein-containing autolysates could be used only in low concentration because of their cytolyzing action); thymine, 10^{-4} M; yeast extract plus 10^{-4} M thymine; combined thymine, xanthine, adenine, uracil and guanine, all at 10^{-4} M; thiamine · HCl, 1 mg %; choline, 5×10^{-4} M; intact and hydrolyzed pentose nucleic acid (from yeast) and desoxypentose nucleic acid (thymus); various amino acids at 10^{-3} M, glycine, cysteine, cystine, arginine, lysine, methionine, proline, hydroxyproline, tryptophane, histidine, threonine, leucine, isoleucine, tyrosine, valine, serine, alanine, glutamic acid, aspartic acid, β-alanine, and α-amino, n-butyric acid; also 10^{-3} M solutions of glutamine, glutathione, and formic acid plus glycine.

The failure of these materials may be interpreted as indicating that the substance required by the cells, if such exists, was not added or that it was impossible by adding it to the medium to achieve adequate intracellular concentrations.

SUMMARY

1. Bis(β-chloroethyl)methylamine (HN-2) and tris(β-chloroethyl)amine (HN-3) over the range 10^{-6}–10^{-4} M produce a reversible block to cell division in *Chilomonas paramecium.*

2. The time course of cell division block and recovery at 17°C. as compared to 25°C. is prolonged in proportion to the slowing of the control growth curve. Recovery is thus correlated with overall metabolic rate.

3. Nitrogen mustards in the solutions used retain inhibitory activity for 10–15 hours.

4. Cells thus blocked resume division in the presence of active mustard. So long as mustard remains in the medium, the cells remain resistant to its action and continue to divide. Fresh solutions of mustard equivalent to the original concentration may even be added without affecting division. If a dose higher than the original one is used, the cells suffer a further inhibition but escape sooner than previously unexposed cells. Removal of the mustard leads to loss of resistance.

5. A consideration of the time course of cell division curves, the effects of temperature on the time course, and the phenomen of resistance development leads to the hypothesis that inhibition of cell division in *Chilomonas* results from a reversible combination of mustard or a derivative with a cell constituent. Continuing generation of this constituent, in the case of combination between mustard and a metabolite, or

piling up of substrate, in the case of combination between mustard and an enzyme, leads to the relief of the inhibition and the development of resistance. (See text.)

6. The time course of turbidity changes in yeast cultures exposed to HN-2 superficially resembles the population changes in chilomonad cultures. It is shown, however, that with yeast, death of a large fraction of the exposed cells followed by repopulation of the cultures by the survivors rather than a reversible block to all of the cells accounts for the turbidity changes noted.

7. One hour's exposure to nitrogen mustard followed by washing of chilomonads produces almost the full inhibitory effect obtainable with a given mustard concentration. This fact has not been reconciled with the reversible inhibition of division in *Chilomonas*.

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SOME EFFECTS OF NITROGEN MUSTARDS ON METABOLISM OF CHILOMONAS PARAMECIUM AND SACCHAROMYCES CEREVISIAE[1, 2]

BETTY PODOLSKY AND JOHN O. HUTCHENS
*Department of Physiology and the Toxicity Laboratory,
University of Chicago, Illinois*

SIX FIGURES

INTRODUCTION

In an accompanying paper we have described the effects of the nitrogen mustards, bis(β-chloroethyl)methyl amine (HN-2) and tris(β-chloroethyl)amine (HN-3), on growth and cell division in *Chilomonas paramecium* and *Saccharomyces cerevisiae.* In this paper we present certain studies of the effects of HN-2 and HN-3 on the metabolism of these same organisms. The two investigations were carried out concomitantly. It was our hope that specific effects of the mustards on metabolic processes would be discovered and would account for the inhibition of cell division and other phenomena which we have described.

It has been reported that the nitrogen and sulfur mustards do not significantly reduce the overall energy yielding reactions of cells (Black and Thomson, '47; Hutchens and Podolsky, '48). Although hexokinase (Needham, '48) and a series of phosphokinases (Gilman and Philips, '46) seem especially sensitive to the action of sulfur and nitrogen mus-

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tards, the expected effects on carbohydrate metabolism are not manifest at doses of mustard which produce profound effects on growth and cell division, e.g., hematopoiesis. A specific effect of HN-2 on desoxypentose nucleic acid synthesis in *Amblystoma* embryos has been reported (Bodenstein and Kondritzer, '48). Villee et al. ('49) found both DNA and RNA synthesis by *Arbacia* eggs to be affected by HN-2.

The nitrogen mustards have been considered to exert their effects through alkylation of enzymes at such loci as $-SH$ or NH_2 groups (Gilman and Philips, '46). In the quaternary nitrogen form of the ethylene imonium ion they can compete with choline for enzymes such as cholinesterase, choline acetylase and choline oxidase (Barron et al., '48). In mammals, doses many times the LD_{50} are required to obtain effects attributable to the cholinesterase inhibition.

Because the effects of nitrogen and sulfur mustards are most striking on rapidly proliferating tissues, we were encouraged to believe that studies made on rapidly growing and dividing cells would disclose biochemical effects which could not be detected using non-growing tissues such as muscle, liver, kidney, etc., or surviving slices from such organs. We have studied respiration, efficiency of assimilation of substrates, the rate of degradation of endogenous substrates, and the synthesis of categories of materials such as carbohydrate, protein and nucleic acids. The results presented below serve largely to eliminate most of these overall processes as the point of attack in the system studied. However, in yeast, the attack on nucleic acid synthesis is striking.

MATERIALS AND METHODS

The cells used were grown, counted, exposed to mustard, and otherwise handled as described in the accompanying paper. Oxygen consumption by *Chilomonas* was measured by the direct method using conventional Warburg respirometers of ca. 20 cm^3 capacity. Carbon dioxide production by yeast was measured by the second method of Dickens and Simer (Dixon, '34), using air as the gas phase. This method permits measurement of both O_2 consumption and CO_2 production. In no case was any O_2 consumption by normal or mustard treated yeast cells detected. Calculations of the thermodynamic efficiency of assimilation of substrates were made by methods previously described (Hutchens, Podolsky and Morales, '48) and were based on the extra O_2 consumption induced by a limited and known amount of substrate. The starch content of the cells was measured as previously described *(ibid.)*.

Nitrogen assimilation by the cells was measured by digestion of trichloracetic acid precipitated and washed cells in H_2SO_4-K_2SO_4 with selenium and copper as catalysts, according to the method of Ma and Zuazaga ('42). The resultant ammonia was distilled according to the above method, the distillate Nesslerized, and the absorption measured at 400 mμ, using a Coleman Junior spectrophotometer.

Nucleic acid content of the cells was measured by several methods, including those of Schmidt and Thannhauser ('45), Schneider ('45), Ogur and Rosen ('49) and Stumpf ('47). The first three methods gave essentially identical values for total nucleic acids. However, in our hands, the various procedures gave widely different values for desoxypentose nucleic acid. Both *Chilomonas* and yeast contain ca. 90% of their nucleic acid in the form of pentose nucleic acid. We finally chose to measure only total nucleic acid using a modification of the method of Ogur and Rosen in which the total nucleic acid was extracted for 20 minutes at 70°C. in 5% perchloric acid and the absorption of the extract measured at 260 mμ in a Beckman Model DU spectrophotometer. Previous to the extraction, the cells were fixed in 70% ethanol containing 0.2% perchloric acid, washed with 95% and then absolute ethanol; extracted twice with 3:1 ethanol-ether at 60°C., washed in absolute and then 95% ethanol, and finally washed twice with cold 2% perchloric acid.

EXPERIMENTAL RESULTS

1. Respiration in Chilomonas. In a series of preliminary experiments we studied the effects of various concentrations of HN-2 and HN-3 on the respiration of chilomonads during the first three hours following exposure. Table 1 presents a typical experiment using HN-3.

It will be seen that concentrations of HN-3 which produce profound inhibition of cell division ($< 1.7 \times 10^{-4}$ M HN-3) cause only an insignificant suppression of respiration.

TABLE 1

Effect of HN-3 on oxygen consumption of Chilomonas

CONC. HN-3 (moles/l)	OXYGEN CONSUMPTION (% CONTROL)			
	1st. hr.	2nd hr.	3rd hr.	Total
1.9×10^{-5}	95	99	101	99
5.7×10^{-5}	97	98	90	95
1.7×10^{-4}	86	92	75	85
5.0×10^{-4}	61	45	35	47

Because division of *Chilomonas* proceeds with only slight inhibition until 30–50% of the cells have divided (i.e., for 2–4 hours), we felt it worth while to study the effects on respiration over a more extended period. The experiment presented graphically in figure 1 shows the effects of 1.1×10^{-5} M and 3.3×10^{-5} M HN-2, concentrations which produce a profound block to cell division. To obtain these data, batches of cells were exposed and handled as in experiments on the time course of cell division. Because growth does not proceed normally in respirometers (Hutchens, Podolsky and Morales, '48), probably because of low CO_2 tensions, new batches of cells were taken every three hours to measure the rate of respiration. As will be seen from figure 1, the rate of O_2 consumption per original cell was the same in both concentrations of HN-2 as in the controls. Because division was blocked three hours after exposure, the rate of O_2 consumption per cell present rose in the HN-2 exposed cells. It seems unlikely that interference with O_2 consumption *per se* is responsible for inhibition of cell division.

2. Efficiency of assimilation of acetate by Chilomonas. Failure of an agent to inhibit respiration does not necessarily indicate that it is failing to affect necessary energy capture

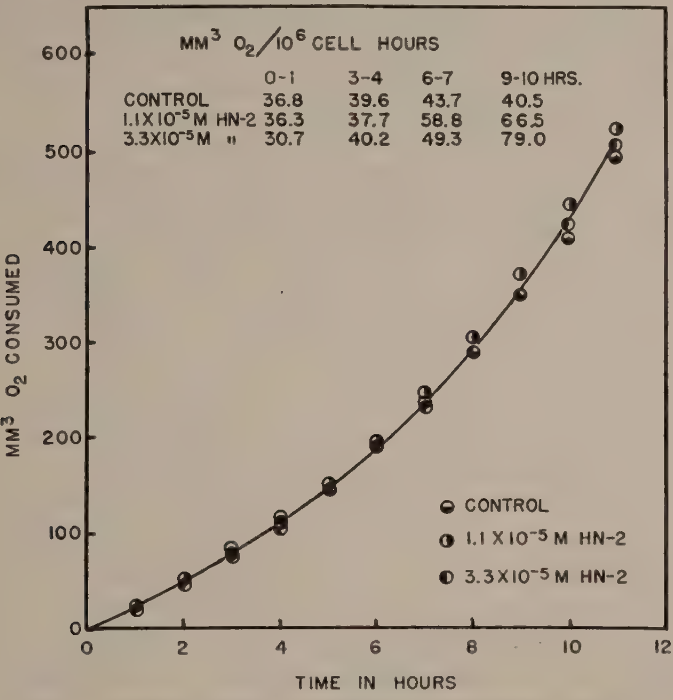

Fig. 1 Effect of HN-2 on respiration in *Chilomonas* at 25°C.

for cell processes. Indeed, agents such as the nitro- and halo-substituted phenols actually stimulate respiration while uncoupling oxidative generation of high energy phosphate bonds (Loomis and Lipmann, '48). The effects of these agents in blocking cell division are well known (see Clowes, '50, for summary review).

We therefore studied the efficiency of assimilation of acetate as a substrate by *Chilomonas*, measuring the extra O_2 consumption induced by a limited but known amount of added acetate (Hutchens et al., '48). Table 2 shows the result of a typical experiment.

As will be seen from table 2, the efficiency of assimilation is not decreased by HN-2. Under these conditions the chief product of synthesis is starch. The question might be raised as to whether efficiency of protein and fat synthesis is affected. Evidence to the contrary will be found in the sections below which indicate that protein and nucleic acid synthesis in these cells is unimpaired.

TABLE 2

Efficiency of acetate assimilation in Chilomonas three hours after exposure to HN-2
(4 µmoles acetate added)

	% ASSIMILATION
Control	67.5
1.1×10^{-5} M HN-2	68.0
3.3×10^{-5} M HN-2	69.8

3. *Effect of HN-2 on synthesis of starch by Chilomonas.* We had several reasons for doubting that the synthesis of starch by *Chilomonas* was inhibited by the nitrogen mustards. The appearance of the iodine stained cells under the microscope indicated that they were abundantly filled with starch. As described above, the overall respiration of the cells was uninhibited and the efficiency of acetate assimilation (chiefly to starch) was not lowered. To confirm our suppositions, we analyzed chemically for the starch synthesized by chilomonads in the presence of 1.1×10^{-5} M and 3.3×10^{-5} M HN-2.

It will be apparent from figure 2 that the synthesis of starch by chilomonads is not inhibited by these concentrations of HN-2. In fact it is definitely accelerated between 5 and 10 hours after exposure. This finding was confirmed in a repetition of the experiment. We are at a loss to explain the increase in rate of starch synthesis since respiration does not increase. Nitrogen deprivation is known to increase the efficiency of acetate assimilation (Blum et al., '51). Inhibition of nitrogen utilization might produce effects like nitrogen deprivation. However (see below), such minor inhibition of protein and nucleic acid synthesis as occurred in the experiments in which these materials were measured was not evident until 7.5–10 hours after exposure to these concentrations of HN-2.

4. Fermentation in yeast. Unlike chilomonads, yeast exhibits a progressive inhibition of respiratory activity following exposure to the nitrogen mustards. As we have shown in the accompanying paper, the ability of the yeast cells to produce colonies following exposure to HN-2 falls off with time. It was of interest to compare the rate of growth of the cultures with the rate of fermentation. Such a comparison is made in table 3.

STARCH/10^6 CELLS AS % OF CONTROL VALUE

HOURS	2.5	5.0	7.5	10.0
1.1×10^{-5}M. HN-2	112	158	232	252
3.3×10^{-5}M. HN-2	117	166	295	386

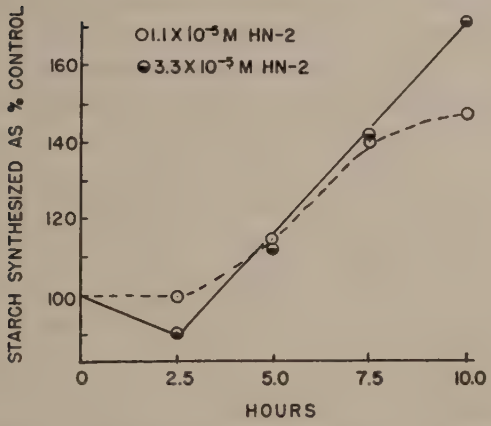

Fig. 2 Effect of HN-2 on the synthesis of starch in *Chilomonas* at 25°C.

It is apparent that these two functions decrease concurrently. Inasmuch as different cultures on different days were used for the growth studies and the various measurements of the fermentation rate, we can attribute no signifi-

TABLE 3

Effect of 1.1×10^{-5} M HN-2 on rate of CO_2 production and growth rate in yeast at 37°C.

HOURS	0.5–1.5	3.5–4.5	5.5–6.5	9.5–10.5	12.5–13.5
Rate of CO_2 production as % of control rate	88.5	75.2	21.3	6.4	5
Growth rate as % of control	97	49	33	11	7

cance to the fact that the rate of fermentation appears to fall off before the rate of growth.

5. *Effects of nitrogen mustards on metabolism of endogenous substrates.* In early experiments in which we studied the effects of HN-3 on synthesis of carbohydrate and protein, we employed relatively high concentrations of HN-3 and depressed the rates of synthesis, particularly of protein. As will be shown later, such inhibitions need not necessarily

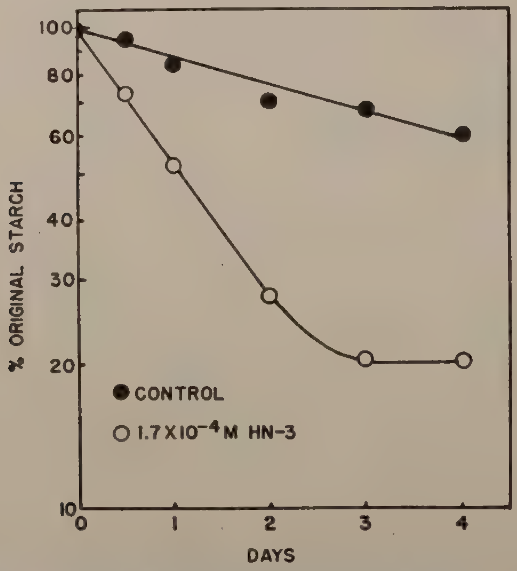

Fig. 3 Effect of HN-3 on the breakdown of stored starch in *Chilomonas* (cells in medium containing no nitrogen or carbon source) at 25°C.

accompany inhibition of cell division. At the time, however, they led us to attempts to distinguish between impaired anabolic and accelerated catabolic processes. We studied the effects of exposure to HN-3 on the rate of breakdown of carbohydrate and protein by chilomonads washed free of exogenous carbon and nitrogen sources. The cells were exposed to the specified concentration of HN-3 for one hour, then washed again to remove the HN-3. The results as regards starch disappearance are shown in figure 3.

As will be seen from figure 3, the control cells lost their starch at a rate of ca. 10% per day. Starch disappeared from the HN-3 treated cells, on the other hand, at a rate of ca. 50% per day. The curve describing the time course of loss of starch from the HN-3 treated cells flattens out at 2–3 days. This is an artifact resulting from the fact that some of these cells cytolyzed, liberating starch plastids into the medium. These plastids were included in the samples taken for analysis. By the end of three days the intact cells contained no starch detectable by staining with Lugol's solution.

In this same experiment, ammonia production was followed as a measure of protein breakdown. None was detectable in cultures either of control or HN-3 treated cells at 24 hours. By 48 hours the control cells had produced 1.3 μg of ammonia N/10^6 cells (ca. 5% of the total cell nitrogen). The HN-3 treated cells had produced 1.83 μg/10^6 cells. At 72, 96 and 144 hours, the ammonia N productions were 2.2, 3.3, and 5.0 μg/10^6 cells respectively in the control cells. Corresponding values in the HN-3 treated culture were 4.4, 6.1, and 9.2 μg/10^6 cells. Therefore, following exposure of chilomonads to this relatively large dose of HN-3, the rates of breakdown of both carbohydrate and protein are accelerated.

We sought to learn whether these increased rates of breakdown of endogenous materials were accompanied by an increased respiratory rate. Figure 4 shows the results of a typical experiment. It is apparent that over a period of days, the HN-3 treated cells consume more O_2 than the controls. It is important to note, however, that this does not result from a stimulation of O_2 consumption above the original control level. What happens is that the rate of O_2 consumption in the control cells declines steadily as soon as they must depend on endogenous stores. As has been shown (Hutchens, '51), this decline starts rather abruptly about two hours after the cells are washed. The rate of O_2 consumption by the HN-3 treated cells also declines, but more slowly. The net effect, if total oxygen consumptions are compared, is to indicate stimulation of the HN-3 treated cells. We have been able to show that a similar situation obtains as regards glycolysis in *Chilomonas.*

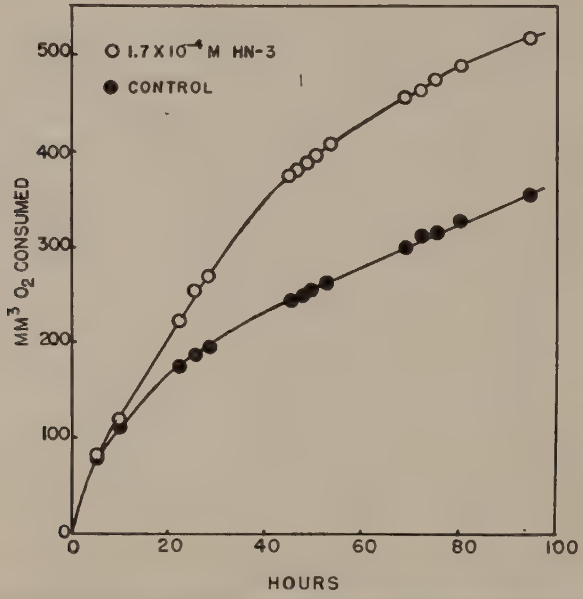

Fig. 4 Effect of HN-3 on endogenous oxygen consumption in *Chilomonas* at 25°C.

Figure 5 shows the rate of glycolysis by normal and HN-3 treated (1.7×10^{-4} M HN-3) chilomonads over a 7-hour period. The conditions of the experiment were as follows: The chilomonads were washed and suspended in a salt solution identical with the usual culture solution except that NaCl was substituted for the NaAcetate and $NaHCO_3$ (4.2×10^{-3}

M) was added. Commercial tank N_2-CO_2 (95:5) was used as the gas phase. As in the case of O_2 consumption, it will be seen that the control rate of glycolysis fell off more rapidly than did that of the HN-3 treated cells. That this excess glycolysis is responsible for the excess O_2 consumption cannot be stated, though it seems likely.

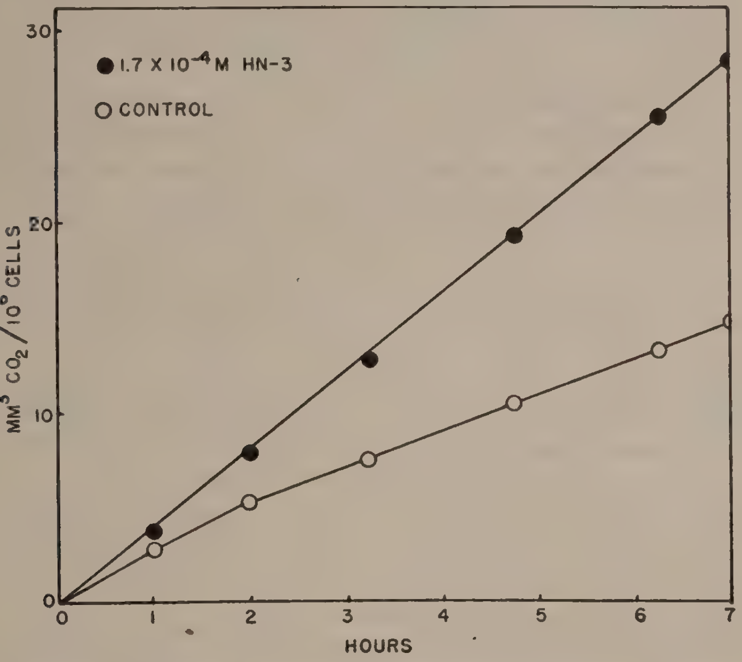

Fig. 5 Effect of HN-3 on glycolysis of endogenous substrate in *Chilomonas* at 25°C.

6. Effects of HN-2 on protein and nucleic acid synthesis in yeast. Effects of nitrogen mustards on nucleic acid synthesis have been previously reported (Bodenstein and Kondritzer, '48; Villee et al., '49). Because of the apparent relation of nucleic acids and protein synthesis, we decided to study simultaneously the effects of HN-2 on synthesis of these two components. As stated in the section on methods, we have been unable to separate adequately pentose- from desoxypentose nucleic acids in yeast and *Chilomonas*. We chose, therefore, simply to extract the total nucleic acids.

Figure 6 shows the time course of synthesis of protein and nucleic acid in yeast following exposure to 3.3×10^{-5} M HN-2. It is seen that there is an early and profound inhibition of the synthesis of both of these materials. By 8 hours, i.e., the time at which increase in optical density of the cultures virtually ceases, the rate of synthesis has fallen to a very low value. It is particularly interesting that the inhibition of nucleic acid synthesis occurs earlier and is more profound than the inhibition of protein synthesis. We therefore suspect that the inhibition of protein synthesis is secondary and consequent on the inhibition of nucleic acid synthesis.

PROT. & N.A./10^6 CELLS AS % OF CONTROL VALUE

HOURS	2.5	5.0	7.5	10.0
PROT.	—	153	—	219
N.A.	100	118	162	174

PROT. & N.A./UNIT O.D. AS % OF CONTROL VALUE

HOURS	2.0	4.0	6.0	8.0
PROT.	—	96	—	78
N.A.	95	65	54	45

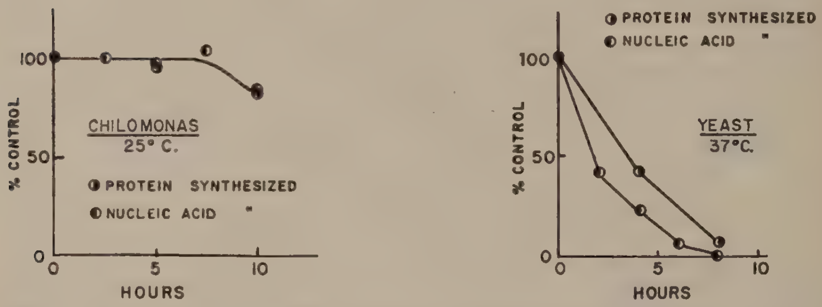

Fig. 6 Effects of 3.3×10^{-5} M HN-2 on protein and nucleic acid synthesis in *Chilomonas* and *Saccharomyces*.

From the measurements of optical density changes in the cultures it was possible to calculate the changes in protein and nucleic acid per light scattering unit. As will be seen from the table above figure 6, both values fall progressively. Whether this indicates that the protein and nucleic acid content of the viable cells falls, it is impossible to say with certainty, since non-viable cells and even cell fragments contribute to light scattering. The decreased nucleic acid/optical density unit at 2 and 4 hours, times at which the majority of cells can still perform at least one more division, supports the idea that nucleic acid synthesis is inhibited but cell division continues in spite of this.

7. Effects of HN-2 on protein and nucleic acid synthesis in Chilomonas. Experiments essentially like those described above involving yeast were performed with *Chilomonas.* Cell counts were made instead of measuring optical densities. As will be seen from figure 6, there is no early inhibition of synthesis of either nucleic acid or protein in these cells. Since cell division is profoundly inhibited after about three hours, this leads to a marked rise in the content of these substances per cell. It is difficult to see how any great significance can be attributed to the decreases in nucleic acid synthesis at 10 hours, since at this time the cells have recovered or are about to recover their ability to divide.

It is of particular interest that in *Chilomonas,* in which the inhibition of cell division is early and profound, nucleic acid and protein synthesis continue. It is of interest also that *Chilomonas* recovers from the inhibition and develops resistance to the HN-2. (See preceding paper.) Yeast, which continues to divide in spite of an apparently decreasing nucleic acid and protein content, does not survive the exposure. It is difficult in the light of these findings to attribute to either the nucleic acids or proteins the responsibility for failure of the cells to divide.

DISCUSSION

It is difficult if not impossible to discuss the results presented in the foregoing sections in terms of any unifying concept of the biological mechanism of action of the nitrogen mustards. In the first place, the two organisms react quite differently following exposure to these agents. Had yeast alone been studied we would probably have concluded that the inhibition of nucleic acid synthesis resulting secondarily in inhibition of protein synthesis accounted for the phenomena observed. It is evident, however, that in *Chilomonas* cell division is inhibited without any profound change in the overall rates of synthesis of nucleic acids, protein or carbohydrate. (Obviously one or more constituents within these larger categories might have been affected without detection.) In *Chilomonas,* respiration and efficiency of assimilation of acetate appear not to be affected. In fact, on the face of things, there seems to be nothing wrong with these cells except that they fail to divide.

In view of the facts presented in this and the accompanying paper, we tend to distrust all of the simplifying and unifying concepts which have been proposed to account for the actions of the mustards. Obviously inhibition of nucleic acid synthesis in yeast is not confined to DNA. Without having separated DNA and PNA we may conclude this, since total inhibition of DNA synthesis could not account for the suppressions noted. Alkylation of sulfhydryl and amino groups, if it occurs under the conditions used here to inhibit cell division, must be highly specific as to sensitivity or margin of safety since so many processes dependent on enzymes bearing these groups proceed uninhibited.

It should be emphasized that these studies of metabolic processes were made using the minimal concentrations of HN-2 and HN-3 which produce observable effects on cell division in *Chilomonas* and yeast. Greater concentrations would undoubtedly have affected synthetic processes (cf. table 1). There seems little room for doubt that the nitrogen mustards attack various systems within cells and that these systems have varying sensitivities to the mustards. In this sense there must be various ''mechanisms of action'' of the mustards, depending on the criteria of damage, e.g., cell division, metabolism, death of cells.

It is not clear whether the present studies are pertinent to a discussion of the effects of mustards on multicellular organisms, particularly those which involve cell death. The fact that yeast cells die after performing several cell divisions, accompanied by impaired synthetic activity, indicates one possible mechanism of killing. That is, continued division with impaired synthesis may lead to exhaustion of a critical cell component. However, cells which stop dividing, such as chilomonads and corneal cells (Friedenwald et al., '47), are killed if the dose of mustard is increased slightly. Exhaustion of a component through cell division cannot be involved in such cases.

SUMMARY

1. The effects of the nitrogen mustards, bis(β-chloroethyl)-methyl amine (HN-2) and tris(β-choroethyl)amine (HN-3) on metabolic processes in *Chilomonas paramecium* and *Saccharomyces cerevisiae* have been studied, using concentrations which profoundly affect cell division in these organisms.
2. HN-2 and HN-3 do not inhibit the energy yielding respiratory activity, i.e., oxidation of acetate by *Chilomonas.*
3. The mustards do not lower the thermodynamic efficiency of assimilation of acetate by *Chilomonas.*
4. The synthesis of starch by *Chilomonas* is not inhibited by the nitrogen mustards.
5. Exposure of chilomonads to 1.7×10^{-4} M HN-3 leads to a rapid attrition of endogenous food stores. Endogenous metabolism of normal cells falls off rapidly after they are washed, but HN-3 treated cells maintain a high rate of glycolysis or oxygen consumption. Starch disappears about 5 times as fast from HN-3 treated as from control cells. Correspondingly, ammonia production from oxidation of cell proteins begins earlier and is more rapid in HN-3 treated cells.
6. Inhibition of sucrose fermentation by yeast exposed to the nitrogen mustards parallels inhibition of ability to produce colonies.
7. The synthesis of nucleic acids and protein by yeast is profoundly inhibited by the nitrogen mustards. Nucleic acid synthesis is inhibited first and most profoundly.
8. Synthesis of nucleic acids and protein by *Chilomonas* is not inhibited during the period in which cell division is most effectively inhibited.

9. None of the observed actions of the nitrogen mustards on overall metabolic processes is adequate to provide a unitary theory of the mechanism of inhibition of cell division.

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CALCIUM DISTRIBUTION AND CONSERVATION DURING THE MOLTING PERIOD IN LIMNORIA LIGNORUM (RATHKE)[1]

FLORENCE M. HARRISON AND ARTHUR W. MARTIN
Department of Zoology and Friday Harbor Laboratories,
University of Washington, Seattle

THREE FIGURES

During the molting process of crustaceans, there is a considerable loss of mineral and organic material. If these substances could be conserved by resorption before ecdysis, they could be utilized for the formation of the new exoskeleton, thereby perhaps reducing the drain on the metabolic processes of the animal.

In the isopod where an interval of time elapses between the molting of the anterior and posterior part of the exoskeleton, the process of conservation is particularly susceptible to experimental analysis. It has been clearly demonstrated in *Ligia exotica* that there is a considerable conservation of calcium. In this form, only approximately 25% of the calcium normally present in the exoskeleton is lost when the animal molts (Numanoi, '34b). In spite of the relatively small amount lost, no significant storage of calcium was demonstrated in the hepatopancreas, blood or remaining soft tissues (Numanoi, '34a, b).

In morphological studies of the molting of isopods, it has been noted that there is an appearance of chalky whiteness in the ventral aspect of the thoracic segments before the on-

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[1] Supported in part by funds for the support of biology and medicine provided by Initiative 171, State of Washington.

set of the molting of the posterior region. Subsequently there is an appearance of a similar whiteness on the pleopods of the abdominal segments before the molting of the anterior region of the body. It was postulated from these observations that the whiteness is due to the formation of deposits of calcareous plates, but no chemical evidence was presented for the presence of calcium (Herold, '13). Nicholls ('31), obtained a positive flame test for calcium with the exoskeleton of the thoracic segments of *Ligia oceanica* and also determined that a gas that would not support combustion was evolved when the white areas of the segments were treated with acid. Both of these tests, however, might be expected to be positive from any piece of calcified exoskeleton.

These formations have been considered to be the sites of storage of the conserved material (Numanoi, '34a). If this is the case, it is necessary to assume a shift of calcium from the posterior to the anterior region and back again during the molting period. Such a shift might be demonstrable by a quantitative analysis of the posterior and anterior parts of the isopod during the various stages of the period. To test this hypothesis, the experiments described below were carried out on the isopod *Limnoria lignorum.*

MATERIAL AND METHODS

The experimental animals were maintained in the laboratory in 10 ml stender dishes on small pieces of wood at a temperature of from 8 to 9°C. Under these conditions molting occurs once every two to 4 months. As is typical for isopods, *Limnoria* molts in two steps. First, the exoskeleton from the 5th thoracic segment posteriad is lost, and then after an interval of from two to 4 days the exoskeleton from the 4th thoracic segment anteriad is lost.

Due to the small size of the animals and the advantage of individual analyses, ultramicroquantitative techniques were employed. All the weighings were made on a Kuhlman microbalance and the mineral analyses were done by the method of Sobel and Hanok ('51), which determines the total calcium and magnesium present. Since the primary concern of this work was the calcium content, it was necessary to analyze for magnesium independently to determine the quantity present. The method of analysis used for the magnesium determination was that of Orange and Rhein ('51). The magnesium present in all samples analyzed ranged from less than 1 to no more than 4 μg. Since the amounts of magnesium present were either so small that they could not be determined accurately or were not large enough to affect the results of the determinations which show the changes in distribution of calcium, the magnesium determinations were not continued for the rest of the work.

TABLE 1

Comparison of the loss of calcium and total dry weight on the molting of the anterior and posterior parts of the exoskeleton

	COLUMN I	COLUMN II
	Ave. % weight loss [1]	Ave. % calcium loss [2]
Posterior molt	14.1, s.d. 3.08	27.3, s.d. 5.88
Anterior molt	8.4, s.d. 1.56	15.2, s.d. 3.38
Total	22.5	42.5

[1] Expressed as per cent of summated dry weight.

[2] Expressed as per cent of the summated calcium.

RESULTS

The animals used in the analyses ranged in dry weight from 205 to 820 μg. The average amount of calcium present in the intermolt animals calculated in terms of dry weights was 14.5%, s.d. 1.25, or about 63 μg in an average animal.

As mentioned previously, there is a considerable amount of mineral and organic material lost with the molting of the animal. The average losses are summarized in table 1.

From Column I of this table it can be seen that 22.5% of the summated body weight (hereinafter used always to include the dry weight of the animal plus the dry weight of the molted material), is lost in the molted exoskeleton. In this same process, as can be seen from Column II, 43.1% of the summated body calcium (hereinafter used always to include the calcium of the animal plus the calcium of the molts), is lost. The molted exoskeleton contains 28.5% calcium, s.d. 5.71, for the posterior part and 26.6% calcium, s.d. 413, for the anterior part.

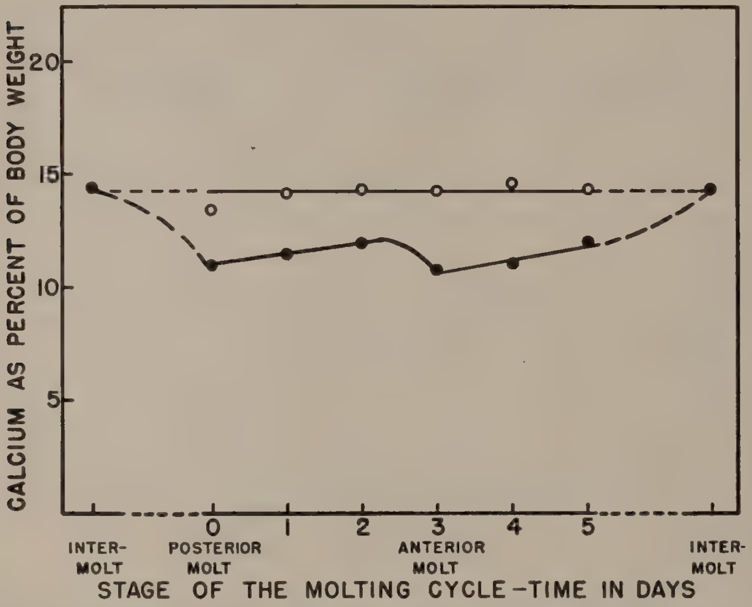

Fig. 1 Changes in the calcium content of *Limnoria* during the molting period.

○ Calcium as per cent summated dry weight of animal.

● Calcium as per cent of dry weight of animal.

From the amount of calcium lost in the molts, it is apparent that there would be a decrease in the percentage of calcium in the molted animal. The data which demonstrate this fact are given by the lower curve in figure 1. However, if the calcium lost in the molts is added to that in the animal, the summated value expressed as per cent of the summated dry weight of the animal and molts remains the same in each stage of the molting cycle as it was in the intermolt animal.

This indicates, since there was no evidence for an increase in dry weight, that there was no net increase in the calcium content by absorption of calcium from the sea water during the period studied. This relationship is represented graphically by the upper curve in figure 1.

In order to determine whether or not there was a shift of calcium from the posterior to the anterior region of the body and vice versa, the changes in distribution of calcium in these regions were studied. Intermolt and molting animals were cut in two between the 4th and 5th thoracic segments. Each part was weighed and then analyzed separately for calcium. The cut was made between the 4th and 5th segments in view of the fact that this is the line of demarcation between the anterior and posterior parts of the exuvia.

TABLE 2

Comparison of the dry weight and calcium distribution in the anterior and posterior halves of the intermolt animal

	COLUMN I	COLUMN II	COLUMN III
	Ave. wt. as % of total wt.	Ave. Ca as % of dry wt. of half	Ave. Ca as % of total calcium
Posterior half	46.9, s.d. 4.44	15.6, s.d. 1.31	50.6, s.d. 3.18
Anterior half	53.1, s.d. 4.44	13.6, s.d. 1.34	49.4, s.d. 3.17

The dry weight and calcium distribution between the two halves of the intermolt animal were determined in order to find out how the cutting of the animal at the place designated affected the distribution of the dry weight. The results show an approximately equal division of each component between the two halves (table 2).

In *Limnoria,* no signs of approaching molting could be found. The onset of the molting was determined by the detection of the molted posterior part of the exoskeleton in the routine twice daily examination of the animals. One group of animals which had just molted this region was designated the series 0-Day. Animals analyzed 24 hours and 48 hours after this stage were placed in the 1-Day and 2-Day series respectively.

The next recognizable stage of the process is the molting of the anterior segments. This usually occurs three days after the detection of the posterior molts. Animals of which the molting of the anterior region had just been detected were placed in the 3-Day series. Animals analyzed 24 and 48 hours after this stage were placed in the 4-Day and 5-Day series respectively.

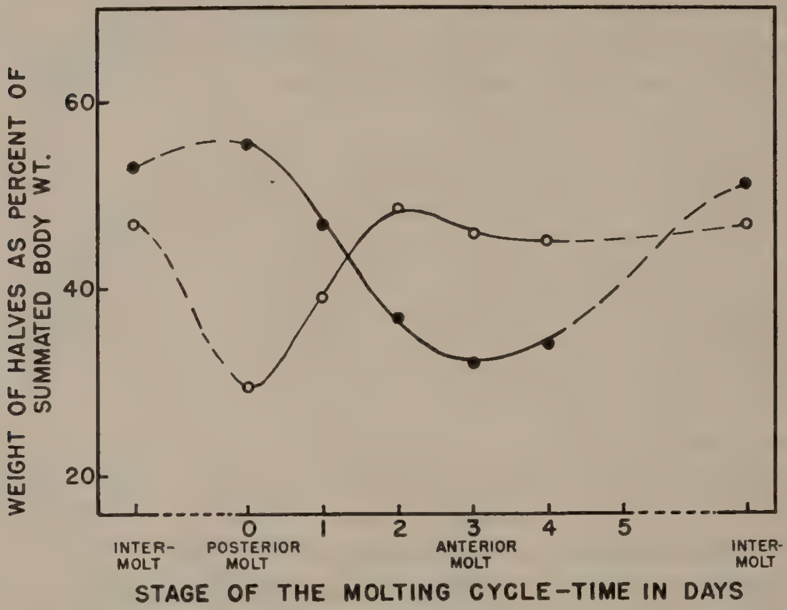

Fig. 2 Changes in the distribution of dry weight between the anterior and posterior halves of the body during the molting period.

● Anterior half of the body.
○ Posterior half of the body.

In the series of animals in the molting period, significant changes were found in the distribution of total dry weight. This is revealed by a comparison (throughout the molting period), of the average weight of the anterior region with that of the posterior region of the animal. The data are presented graphically in figure 2. Each point on the graph is based on a sample containing 6 to 9 animals.

From the graph it is evident that when the anterior and posterior parts of the exoskeleton are molted, there is a decrease in the weight of the half that is undergoing ecdysis. But in addition to this, there is a shift in weight from the anterior to the posterior part of the body after the molting of the posterior part but before the molting of the anterior part of the exoskeleton.

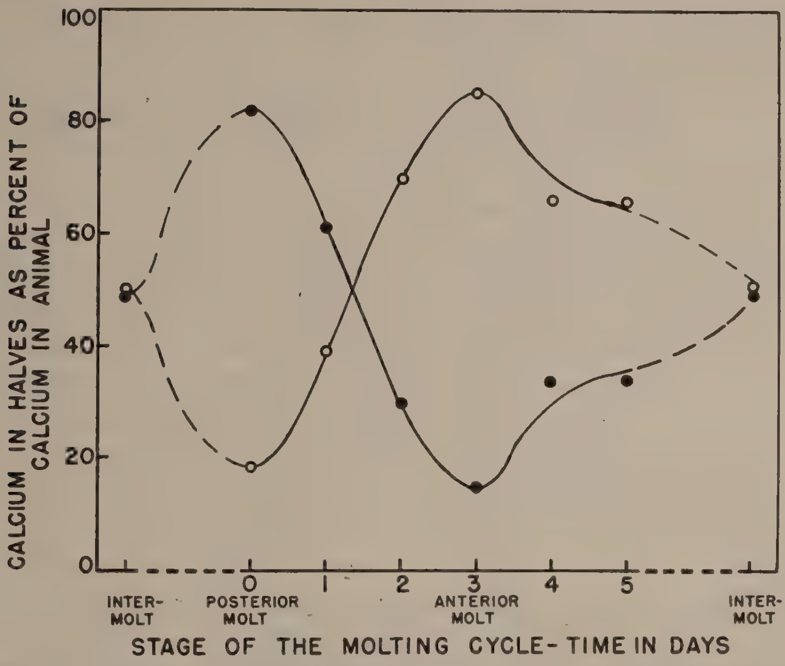

Fig. 3 Changes in the distribution of calcium between anterior and posterior halves of the body during the molting period.

● Anterior half of the body.

○ Posterior half of the body.

The changes in the distribution of the calcium between the two halves can be seen in figure 3 where the values are expressed as per cent of the total calcium of the animal. Since it has been shown that there is no indication of a gain in calcium during the molting period, any change in relative concentration represents either a loss of calcium in the molted tissue or a shift in calcium. From figure 3 it can be seen that the total calcium in the posterior half of the animal changes from 18.1% at 0-Day to 69.9% at 2-Day, a change of about 52%. This represents a shift of about 24 μg of calcium from the anterior to the posterior region in the short interval before the loss of the anterior molt. The reliabilities of the differences between the points corresponding in time were determined by the "t" test of Fisher ('30). These points on the curves are reliably different from each other.

DISCUSSION

The total calcium lost in the molting of *Limnoria* averaged 42.5%. If no calcium is lost by processes other than that in the molting, the amount retained in the body is approximately 57%. It will be recalled from the introduction that approximately 25% of the calcium normally present in the exoskeleton is lost by *Ligia exotica* during the molting process (Numanoi, '34b). Perhaps the better conservation of calcium by *Ligia* can be considered an adaptation to a land environment where calcium is generally less accessible.

From his work on *Ligia*, Numanoi ('34a) concluded that the formation of the chalky whiteness in the thoracic segments and the formation of similar deposits on the pleopods were sites of storage of conserved material. Since he did not determine quantitatively the amount of calcium present in these deposits, he could not know whether the amount present was large enough to be significant as a conservation mechanism. It appears clear from the work on *Limnoria* based upon actual calcium analyses that before the anterior region of the body molts, there is a shift of calcium to the posterior region and that this shift is large enough to be considered a mechanism of calcium conservation.

It appears likely that a similar shift from the posterior exoskeleton to the anterior part of the body might precede the molting of the posterior part, since analyses of the exuviae show only small differences in the extent of calcification. Unfortunately, the shift in distribution of materials could not be determined for the period preceding the molting of the posterior part of the exoskeleton since it was impossible to recognize the molting period until the molting of this region had taken place.

Further analysis of the changes in dry weight indicated that changes in distribution of calcium or calcium carbonate do not account completely for the shift in dry weight from the anterior to the posterior region. It can be calculated that about 11% of the total dry weight materials other than calcium carbonate remaining after the molting of the posterior region are conserved. This is thought to be organic material which has been resorbed from the exoskeleton, but no verification of this hypothesis was attempted.

SUMMARY

1. The dry weight lost in the molting process in *Limnoria* averaged 22.5% of the summated dry weight (dry weight of body plus molts). In the same process, 42.5% of the summated body calcium was lost (calcium of the animal plus molts).

2. The natural boundary between the anterior and posterior molts is at the junction of the 4th and 5th thoracic segments. The anterior and posterior halves of animals cut in two at this juncture are approximately equal in dry weight and contain approximately equal quantities of calcium. During the molting period a series of reciprocal changes take place in the distribution of material between two halves of the body that is interpreted as a conservation of material.

3. Approximately 42% of the calcium remaining in the body after the posterior part of the body has molted was shifted to the posterior region before the anterior region was molted. At this same time, approximately 11% of the dry material other than calcium carbonate was shifted.

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